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Nutrigenomická analýza vlivu diety v průběhu prenatálního a časného vývoje na manifestaci aspektů metabolického syndromu v dospělosti

Nutrigenomic analysis of diet influence in prenatal and early development on metabolic syndrome aspects manifestation in adulthood

Dissertation thesis

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Institute of Biology and Medical Genetics

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List of contents

Abbreviations

5mC	5-methylcytosine
6mA	6-methyladenine
ANOVA	analysis of variance
APE 1	apurinic/apyrimidinic endonuclease 1
ATP	adenosine triphosphate
BAT	brown adipose tissue
BMI	body mass index
bp	base pair
BNST	bed nucleus of stria terminalis
BPA	bisphenol A
BTB/POZ	bric-a-brac, tramtrack, brad complex/poxvirus zinc finger
C	cholesterol
cDNA	complementary DNA
cRNA	complementary RNA
Cys2-His2	two cysteine and two histidine residues binding zinc to stabilize the structure
CM	chylomicron
CO_2	carbon dioxide
CpG	5'- cytosine – phosphate – guanine – 3'

DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol, Clelands agent – enzyme stabilizing agent
dTTP	deoxythymidine triphosphate
dUTP	deoxyuridine triphosphate
DOHAD	developmental origins of health and disease
EDC	endocrine disrupting chemicals
EIF2	eukaryotic initiation factor 2 signaling pathway
EPO	erythropoietin
F1-F3	filial generations 1-3
FAM	6-carboxyfluorescein
FDR	false discovery rate
G-CSF	granulocyte colony stimulating factor
GIP	gastric inhibitory polypeptide
GLP-1	glucagon-like peptide-1
GM-CSF	granulocyte-macrophage colony stimulating factor
GRO/KC	growth-regulated oncogene/keratinocyte chemoattractant
GWAS	genome-wide association study
HDL	high density lipoprotein
HOMA-IR	homeostatic model assessment of insulin resistance

HPA	hypothalamic-pituitary-adrenal axis
HPLC	high performance liquid chromatography
HSD	high-sucrose diet
IAP	intracisternal A-particle
IFN	interferon
IFN-γ	interferon gamma
IGF-1	insulin-like growth factor 1
IL-1α	interleukin 1 alpha, hematopoietin 1
IL-1β	interleukin 1 beta, leukocytic pyrogen
IL-2	interleukin 2
IL-4	interleukin 4
IL-5	interleukin 5
IL-6	interleukin 6
IL-7	interleukin 7
IL-10	interleukin 10, human cytokine synthesis inhibitory factor
IL-12p70	bioactive form of interleukin 12
IL-13	interleukin 13
IL-17	interleukin 17
IL-18	interleukin 18
IRS-1	insulin receptor substrate 1

IUGR	intrauterine growth restriction
IVT	*in vitro* transcription
KATP	ATP-sensitive potassium channel
LDL	low density lipoprotein
M-CSF	macrophage colony-stimulating factor
MCP-1	monocyte chemoattractant protein-1
MetS	metabolic syndrome
MGB	minor groove binder
MIP-1α	macrophage inflammatory protein 1 alpha
MIP-3α	macrophage inflammatory protein 3 alpha
mRNA	messenger RNA
miRNA	micro RNA
mTOR	mammalian target of rapamycin signaling pathway
NAD^+	oxidized form of nicotinamide adenine dinucleotide
NAFLD	nonalcoholic fatty liver disease
NFQ	3’ nonfluorescent quencher
NPY	neuropeptide Y
OGTT	oral glucose tolerance test
PCR	polymerase chain reaction
POMC	proopiomelanocortin

PP	pancreatic polypeptide
PVN	paraventricular nucleus
PYY	peptide tyrosine tyrosine
qPCR	quantitative PCR
RANTES	“regulated upon activation normal T cell expressed and secreted” chemokine
RDA	recommended daily allowance
RGD	rat genome database
RIN	the RNA Integrity Number – RNA quality assessment tool
RNA	ribonucleic acid
RMA	robust multichip average
ROS	reactive oxygen species
RT-PCR	real time PCR
SAM	S-adenosyl-methionine
SEM	standard error of mean
SHR	spontaneously hypertensive rat
siRNA	small interfering RNA/short interfering RNA
SNP	single nucleotide polymorphism
STD	standard diet
T2D	type 2 diabetes
TdT	deoxynucleotidyl transferase

TG	triglyceride, triacylglycerol
TNDM1	transient neonatal diabetes mellitus type 1
TNFα	tumor necrosis factor alpha
UDG	uracil-DNA glycosylase
VEGF	vascular endothelial growth factor
VLDL	very low density lipoprotein
WAT	white adipose tissue
WHO	World Health Organization

Abstrakt

Stoupající prevalence nepřenosných nemocí po celém světě vyzývá ke snaze rozluštit jejich příčiny. Zejména běžné metabolické poruchy zatěžují systémy zdravotní péče a jsou jednou z nejčastějších příčin horší kvality života pacientů. Metabolický syndrom představuje souběh více stavů – dyslipidémie, obezity, hypertenze a zhoršené glukózové tolerance – změněných metabolických fenotypů závislých na genetických a enviromentálních faktorech. Nedávné studie naznačují, že expozice jistým enviromentálním podnětům v průběhu časného vývoje jsou schopné výrazně pozměňovat savčí fenotypy. Výživa jako jeden z významných faktorů ovlivňujících zdraví je přirozeně předmětem výzkumu, zabývajícího se souvislostí parentální diety a fenotypické alterace potomstva. Vývojové počátky zdraví a nemoci se historicky víc zaměřovaly na podvýživu matky, ale s ohledem na současné diety západního typu je důležité orientovat se také na přebytek makronutrientů. Navrhujeme, že je relevantní nejenom množství makronutrientů v mateřské dietě, ale také jejich zdroje, protože můžou zvyšovat riziko onemocnění u potomků. Ukázali jsme, že sacharóza jako alternativní zdroj sacharidů v mateřské dietě má zřetelný dopad na metabolismus potomků v dospělosti, což může být regulováno variací genu *Zbtb16*. V F1 generaci, samci potkanů programovaní v časném vývoji vysokosacharózovou dietou matky vykázali nárůst hnědé tukové tkáně (o 46.5 % u SHR vs. 70 % u SHR-Zbtb16), s potenciálně narušenou funkcí, kterou naznačila transkriptomická analýza. Analýza transkriptomických profilů jater a bílé tukové tkáně také odhalila kmenově specifické rozdíly. Ty kmenově specifické byly zviditelněny nutriční zátěží sacharózou v dospělosti, kdy kongenní kmen SHR-Zbtb16 projevil výraznější inklinaci k fenotypu podobnému metabolickému syndromu než SHR, se zhoršením glukózové tolerance, inzulinémií a zvýšenou hladinou glycerolu (o 15 % u SHR vs. o 46 % u SHR-Zbtb16) v séru. Metabolické efekty programování sacharózou v průběhu časného vývoje byly patrné i v následující F2 generaci potomků. Některé z mezigeneračních efektů se projevily odlišně než v F1, zejména vyšší lačná glykémie a zvýšené hladiny HDL. Sacharóza jako zdroj sacharidů v mateřské dietě může mít významný dopad na rozvoj fenotypu podobnému metabolickému syndromu ve dvou generacích potomků, což je do jisté míry modulováno mutací genu *Zbtb16*.

Klíčová slova: metabolický syndrom, výživa, Zbtb16, DOHaD, sacharóza, hnědý tuk

Abstract

The rising prevalence in noncommunicable diseases worldwide calls for the effort to determine their underlying causes. Common metabolic disorders in particular overwhelm the healthcare systems and are a one of the leading causes of poor quality of life of patients. Metabolic syndrome is represented by concurrence of several conditions - dyslipidaemia, obesity, hypertension or impaired glucose tolerance - altered metabolic phenotypes related to genetic and environmental factors. Recent studies suggest that early-life exposure to certain environmental stimuli is particularly capable of changing the mammalian phenotypes. Nutrition, as one of the major factors influencing health, is naturally a focus of research, which studies the link between parental diets and phenotypic alterations in offspring. The developmental origins of health and disease were historically more focused on maternal undernutrition, it is, however, more important to focus on surplus of macronutrients considering the westernization of modern diets. We propose the relevancy of not only the amount of macronutrients in maternal diet, but also their sources, as they may increase disease risk in offspring. Here we report, that sucrose as an alternative carbohydrate in maternal diet, has a marked impact on metabolism of the offspring in adulthood, which can be modulated by *Zbtb16* gene variation. In F1 generation, male rats programmed in early life by maternal high-sucrose diet showed an increased proportion of brown fat (by 46.5% in SHR vs. 70% in SHR-Zbtb16), which also showed distinct a transcriptomic profile pointing to a compromised function. The transcriptomic profiles of liver and white fat tissue also revealed strain-specific differences. The response variations were illustrated by a nutritional challenge with sucrose in adulthood, when congenic strain SHR-Zbtb16 displayed more significant inclination towards metabolic syndrome-like phenotype than SHR, with deterioration of glucose tolerance, insulinemia and elevated levels of glycerol (by 15% in SHR vs. 46% in SHR-Zbtb16) in serum. The metabolic effects of early life programming with sucrose were apparent in subsequent F2 generation of offspring. Some of the intergenerational effects manifested differently than in F1, in particular higher fasting glycaemia and increased levels of HDL. We show that sucrose as a source of carbohydrates in maternal diet can have significant impact on development of metabolic sydrome-like phenotypes in two generations of offspring, which is to an extent modulated by a mutation in *Zbtb16* gene.

Keywords: metabolic syndrome, nutrition, Zbtb16, DOHaD, sucrose, brown fat

1. Introduction

The global public health status in the 21. century is characterized by a massive increase in prevalence of noncommunicable diseases. More individuals than in any time in history meet diagnostic criteria for heart disease, diabetes and chronic lung disease, leading to almost 70% of all mortality worldwide, according to WHO. The prevention and control of these diseases should be one of the imperatives in public health sector in the present moment. Evidence from animal models, as well as epidemiological human studies, has led to the hypothesis, that nutrition in early life can significantly impact the long-term risk of obesity, cardiovascular disease and type 2 diabetes [1]. The concept of metabolic programming links early intrauterine environment exposures during pregnancy with programmed changes in gene expression that alter offspring development and growth. These alterations can result in metabolic syndrome-like phenotype in adults [2], and are able to persist in subsequent generations even in the absence of environmental factors which induced these changes in the first place.

Nutrition is one the most important environmental factors influencing human health and well-being. With the historical shifts from famine to feast in many populations, the alterations in resulting phenotypes are suspected to contribute to the epidemic in noncommunicable diseases in the present. Despite the relevance to modern human diets, studies investigating altered maternal nutrition are rarely focusing on the particular sources of carbohydrates. Sucrose and fructose, used as sweeteners, are increasingly more present in diets and are a significant contribution to the daily caloric intake of all age groups. If we accept the fact that nutrition and lifestyle choices significantly impact the health of adults, we must admit the possibility, that it has to have an effect in developing mammalian organisms as well. Moreover, the influence should be considered even more impactful, as the biological and hormonal systems are only being developed and set for postnatal function. The setting of mammalian systems decides at what capacity the organism will be able to handle the load of different forms of stress. Developmental plasticity tells us that as mammals, we are supposed to be customized for our postnatal experience by detection of the surrounding environment. The unprecedented abundance in sugar in our diet can, after all, affect our phenotype in unparalleled ways.

2. Aims of the study

This PhD thesis aims to test a hypothesis that genetic factors represented by a specific variant of *Zbtb16* gene influence susceptibility to epigenetic nutritional stimuli in early development and whether they have an impact on manifestation of metabolic syndrome in adulthood. The main aim of the Thesis includes studying environmental interactions of the variant *Zbtb16* gene and high-sucrose diet also the effects of various diets in the course of early development on postnatal health of the rat offspring.

Partial aims of the thesis:

1. Testing of hypothesis that high-sucrose diet feeding in pregnancy and lactation has an impact on metabolic syndrome components in adult offspring depending on the presence of variant *Zbtb16* gene in SHR-Zbtb16 novel congenic strain of rat,

2. Testing of hypothesis that nutritional challenge with high-sucrose diet in adulthood would exacerbate the different responses in SHR vs. SHR-Zbtb16,

3. Testing the hypothesis that high-sucrose diet feeding effects transfer to the F2 generation of offspring,

4. Testing the hypothesis that pharmacological challenge with dexamethasone exacerbates the different responses of both strains,

5. Testing the hypothesis that a specific time window for high-sucrose diet feeding is important to have an impact on offspring health and that the effects also transfer to the F2 generation of SHR-Zbtb16 offspring.

3. Literature review

3.1. Metabolic syndrome

Metabolic syndrome (MetS), previously called “Syndrome X”, describes concurrent metabolic abnormalities that increase the risk of coronary heart disease and type 2 diabetes. The diagnosis requires simultaneous presence of at least three pathophysiological characteristics – obesity, hypertension, insulin resistance, elevated fasting plasma glucose, type 2 diabetes (T2D) and atherogenic dyslipidemia [3; 4] or treatment.

Obesity (BMI ≥ 30 kg/m2) is defined by the World Health Organization (WHO) as an excessive adipose tissue accumulation sufficient to impair health. Pathological weight gain in central part of the body (central visceral adiposity) contributes to increase in free fatty acids in circulation, which are able to interfere with insulin signaling and beta cell function via their metabolites. In 2016, more than 1.9 billion adults were overweight (650 million obese), which led to 1 in 5 deaths in the world connected to obesity [5; 6] (WHO Obesity and Overweight Fact Sheets, 2018). It is important to note, that this category of MetS is not met only when an individual is clinically obese. Visceral adiposity seems to have definitive impact on development of metabolic pathologies and is the criterion is therefore met with elevated waist circumference, characterized by ≥ 102 cm in males and ≥ 88 cm in females [7; 8].

Equivalently to obesity, type 2 diabetes is highly prevalent, with 463 million people worldwide suffering from this condition in 2019 (IDF Diabetes Atlas 9th edition 2019). T2D is defined by impaired glucose homeostasis, beta cell dysfunction and insulin resistance in metabolic tissues, which is often induced by obesity. Insulin resistance in peripheral tissues leads to hyperinsulinemia, which can deplete beta cells and cause persistent hyperglycemia. Overall, T2D is a complex multifactorial metabolic disorder influenced by lifestyle, environment and genetic factors [9].

The major component of atherogenic dyslipidemia is elevation of apo B-containing lipoproteins such as low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL). Along with elevated triglycerides and reduced high-density lipoprotein (HDL) cholesterol, these abnormal lipid levels associate strongly with atherosclerotic cardiovascular disease and contribute to development of metabolic syndrome [10; 11]. The elevation of apo B-containing lipoproteins is crucial for development of atherosclerosis, which rarely develops only in the

presence of other risk factors [12]. Lipoproteins in circulation filter into the arterial wall and are incorporated into macrophages (foam cells), which then can degrade and form a fibrous plaque [13]. Rupture of plaque and consecutive thrombosis precipitation can lead to acute cardiovascular event, e. g. myocardial infarction or stroke [12; 14; 13; 15].

Obesity and metabolic syndrome seem to come hand in hand with hemostatic system abnormalities such as endothelial dysfunction, enhanced coagulation, impaired fibrinolysis and platelet dysfunction. Notably, individuals with metabolic syndrome have increased baseline platelet reactivity and are at higher risk for venous thrombosis [16; 17].

Raise in blood pressure is often a result of concurrence of several factors, including release of angiotensinogen from adipose tissue, enhanced renal reabsorption of sodium and activation of renin – angiotensin – aldosterone system and sympathetic nervous system [18; 19]. Hypertension present in individuals with metabolic syndrome, as well as atherosclerosis doubles the risk for stroke [20].

It is important to note that global expenses for treatment of "globesity" (WHO), notably metabolic syndrome and its individual contributing components present a major economic burden on healthcare and it is therefore necessary for scientific inquiry to work towards identification of underlying mechanisms, causes and prevention of these pathophysiological states in the future. The collaborative effort of science and medicine should effectively lower these widespread causes of morbidity and mortality, simply by understanding the causes and implementing findings in clinical care. Unfortunately, the development of metabolic syndrome prevalence in last few years points to the contrary and calculated trends illustrate an ominous threat to global public health, with predictions of even greater increase in obesity in all age categories, as well as rampant prevalence of cardiovascular diseases.

3.2. Nutrigenetics

Nutrigenetics and nutrigenomics are fields of genetics focused on nutrition, specifically the effect of genetic variation on dietary response and the role of nutrients and other food compounds in gene expression. The importance of this particular field of science is significantly supported by three factors:

1. nutrient bioavailability and metabolism are affected by great genomic diversity between ethnic groups and individuals
2. the existence of vast differences in food or nutrient availability and choices is dependent on economic, geographical and cultural factors
3. malnutrition (deficiency or excess) can change gene expression and genome stability which can lead to altered phenotypes and possibly disease [21-25].

Nutrients and food bioactives exert biological effects which depend on various physiological processes. Absorption, transport, biotransformation, uptake, binding, cellular mechanisms of action all involve genes, which can carry polymorphisms potentially altering their function and ultimately the physiological response to dietary compound [26]. Genes have also been reported to influence food preferences by affecting pathways connected to sensory or reward systems [27].

The widely accepted assumption that individuals have the same nutritional requirements are often supported by dietary guidelines constructed merely to prevent deficiency diseases. Although nutritionists recognize differences in needs of children versus adults, males compared to females, the recommended daily allowance (RDA) or safe upper limits are designed for the general population. These standards are not optimized for various genetic subgroups which possibly differ critically in the activity of transport proteins, enzyme levels and activities and would therefore benefit from personalized nutrition. Genetic variation across human genome is too complex for easy one-fits-all solutions. Common genetic polymorphisms can occur in up to 40-50% of the population, and even polymorphisms with at least 1% occurrence such as single nucleotide polymorphisms (SNPs), provide a vast platform for genetic variation in human populations, as there have been reported over 10 million SNPs in public databases. Not only nucleotide insertions or deletions, but also copy number variants can modify personal response to diet, simply because those variants that do, have significant effects on the structure or function of the gene product. The most common approach to the identification of gene variants modifying effects of dietary factors or influencing the food preferences is a process of selection based on known or putative function, otherwise known as candidate gene study. Analyzing individual SNPs or combinations of SNPs (haplotypes) can provide more knowledge on individual variability in response to bioactive food compounds and identify relevant nutrient-gene interactions. Understanding of these complex issues not only benefits the individuals seeking dietary advice but can help improve public health recommendations [21-25].

The focus of nutrigenetics involves a broad spectrum of genes related to nutrient metabolism and pathways requiring micronutrients as cofactors. An important aspect of nutrient-gene interaction studies is epigenetics, as diet is able to either on its own or in combination with other environmental factors cause epigenetic changes that activate or repress transcription of certain genes. DNA methylation is one of the most important epigenetic mechanisms in mammals and is solely dependent on methyl donors e. g. folate, vitamin B12, choline and methionine. Deficiency of methyl donors in diet leads to lack of methylation or inhibition of DNA methyltransferases and its effects are described in detail in the section 3.3.2.3.4 [26]. Dietary effects on genome stability (DNA damage), epigenome alterations (DNA methylation), RNA and micro-RNA expression (transcriptomics), protein expression (proteomics) and metabolite changes (metabolomics) are all disciplines used in the field of nutrigenomics to study health outcomes of altered nutritional exposure and can also be used to study a disease trajectory in relation to nutrition [26].

Gene-diet interactions are described as able to modulate the effect of dietary compounds on a particular phenotype by a genetic polymorphism [28]. To this day, research studies have reported a vast amount of findings using nutrigenetic knowledge in the last decades. With focus on prevention, studies have shown gene variants connected to weight loss success with personalized diets [29; 30] and expanded the understanding of the roles of specific variants of genes involved in lipid metabolism which may accelerate the development of neurodegenerative diseases such as Alzheimer and Parkinson's [31]. Copy number variation seems to be an additional variable that influences response to nutrients, however previously overlooked. Studies observed that up to 25% of individual variation in response is dependent on copy number variation [32] e. g. increase in amylase gene copy number was associated with an increased enzymatic activity and starch digestion [33]. Although there is a potential of dietary compound effect modulation, vast number of SNPs analyzed in number of studies show not significant results of genotype-diet interactions of measurable functions such as weight loss [34].

Environmental factors that interact with specific genes are a very broad and complex concept, spanning from toxic exposures and drug consumption to socioeconomic status. However, food intake is the environmental factor to which we are all exposed permanently and therefore dietary habits have the most potential for modulating gene expression during a person's lifespan [28].

3.3. Early life epigenetic programming and developmental plasticity

One of the most important abilities of living organisms is perception, evaluation and adaptation to surrounding environments. Adoption of appropriate responses increases the chance of survival and reproduction, and maintaining a memory of such responses allows for coping with similar conditions when encountered later in life [35-37]. An inherited trait or a behavioral adaptation are usually beneficial and help adjust to the changing environment, however, a mismatch between external conditions and adaptation can be maladaptive and not fit the offspring's environmental demand [38-40]. The biological mechanisms underlying adaptations to the environment are complex and presumably mediated by non-genomic processes such as epigenetic mechanisms [41-43].

3.3.1. Epigenetic inheritance

Organisms display physiological and also behavioral alterations in response to changing environments and they are able to pass these changes on to their progeny [44]. Epigenetic inheritance systems represent a potential mechanism by which parents could transfer information about the environment that they've experienced to their offspring [45]. This information transfer can be adaptive and help the offspring's survival in the environment; however, it can also elicit responses that are not adaptive and represent merely the direct effect of the disruptor's action. The inheritance of acquired traits is commonly referred to as "Lamarckian" after evolutionary theorist Jean Baptiste de Lamarck. It is however important to mention that both Charles Darwin and J. B. Lamarck believed in the possibility that acquired traits can be inherited [45].

3.3.1.1. Mechanisms of epigenetic inheritance

Epigenetics generally refers to the long-term regulation of gene expression and function induced by environmental factors without a change in DNA sequence [44]. Certain epigenetic changes can be transmitted from parents to their offspring, allowing organisms to transfer adaptive and non-adaptive information related to the ancestral environment [44].

Research suggests three main models of transmission of environmentally induced alterations, depending on the number of subsequent generations that express acquired traits, but weren't exposed to the environment that triggered the epigenetic change [46-48]:

1. intergenerational effects – F1 generation only (developing embryo and its germline) affected by in utero or paternal exposures to environmental stress [49-51],
2. multigenerational effects – from F1 to F2 generation [52],
3. transgenerational effects – more than three generations [53-55].

Regarding the adult-onset disease, epigenome modification of the developing organ can be critical for the exposed individual, however only the reprogrammed germline is required to transmit this phenotype transgenerationally [53; 56]. If the F0 gestating female is exposed to an environmental factor, the developing F1 embryo is in this regard directly exposed. This embryonic exposure to environmental factors such as nutrition or toxic substances can influence disease or abnormal phenotypes in the F1 generation, but as a result of direct exposure, it is not considered a transgenerational phenomenon [57]. Furthermore, an exposure of gestating F0 female directly affecting the F1 embryo also exposes present F2 generation germline. During embryonic gonadal development, the germline undergoes critical programming of its epigenome that subsequently impacts the progeny from those germ cells. Should the alternations in F2 generation be described as multi-/transgenerational is debatable. The phenotype of F2 generation can be a result of abnormality generated by the direct exposure (not transgenerational) or as a result of permanent reprogramming of the germline epigenome (transgenerational), which can only be tested if the next F3 generation is produced and further analyzed [57].

The mechanisms underlying parental environment-induced epigenetic inheritance include DNA methylation, histone modifications, and production of non-coding RNAs. Since epigenetic information undergoes extensive reprogramming, which occurs after the fertilization and during gametogenesis in the germline, we consider the transferred epigenetic information as that which escaped this resetting and carries the environmental information [58]. The central question of epigenetic inheritance studies is, therefore, how are the patterns of epigenetic modifications established and maintained to escape two waves of epigenetic reprogramming [44].

DNA methylation in mammals occurs mainly at the palindrome dinucleotide sequence 5'CpG3' at the 5 position of cytosine (5mC) [59] and is enzymatically catalyzed by DNA methyltransferases DNMT3A, DNMT3B and DNMT1 [60; 61]. During DNA replication, the daughter strand carries the newly generated CpG sequences from methylated CpG in the parental strand, which is copied by DNMT1 [62-64], whereas DNMT3A and DNMT3B establish de novo methylation patterns during development [65]. DNA methylation in the germline is eliminated during gametogenesis and post fertilization, however extensive, this erasure is not complete [66-68]. Methylated states that escape the erasure following fertilization can be maintained at differentially methylated regions of imprinted genes and in repetitive sequences such as the retrovirus element intracisternal A-particle (IAP) [69] which then carry the epigenetic memory to the progeny. Still, the majority of methylcytosine in sperm is converted to hydroxymethylcytosine by the Tet3 enzyme after fertilization. The maternal cytosine methylation is protected from hydroxylation and therefore demethylation by the PGC7/Dppa3/Stella protein, which also acts in maintenance of paternally imprinted regions in sperm via binding to heterochromatic histone mark H3K9me2 [70].

Histone modifications occur through chemical modification of the N-terminal tails of histones and include methylation [71], acetylation [72], phosphorylation [73], sumoylation [74] and ubiquitination [75]. Histone modifications play a role in the inheritance of acquired phenotypes in Caenorhabditis elegans, Drosophila melanogaster and Schizosaccharomyces pombe [76; 50; 77], from which the DNA methylation is mostly absent. Mammals differ from invertebrate organisms, particularly in their epigenomes [78; 79], however mammalian gamete display its own unique chromatin states in which histones are mostly cleared and replaced by protamines in mature sperm [80]. Evidence suggests that despite the low percentage of haploid genome in humans packaged into nucleosomes (10% in mature sperm), certain genes maintain the H3 lysine 27 trimethylation (H3K27me3) histone modification at their promoters [81] and therefore histone modification can transfer epigenetic memory from one generation to the next [44].

Non-coding RNAs such as miRNAs and siRNAs were shown to be involved in parental environment-induced epigenetic inheritance. Studies have demonstrated recently that tRNA-derived small RNA fragments in sperm act as a paternal epigenetic factor and contribute to intergenerational inheritance of diet-induced metabolic disorders, which concurs the RNA

content found in sperm together with highly condensed nucleus and little cytoplasm, suggesting RNAs may be involved in transmission of acquired traits from father to offspring [82; 83].

A number of environmental factors have been shown to induce epigenetic changes in different genes [84; 85; 53; 86]. A lot of DNA methylation changes are not stable and therefore, not heritable, but imprinted genes maintain a DNA methylation pattern in a heritable manner [84; 87; 88]. Since the most sensitive developmental periods to environmental exposures are embryonic and early postnatal periods [56; 89; 90], it is likely so due to the occurrence of altered developmental processes which change subsequent organ development and function [57].

3.3.1.2. Epigenetic inheritance in nematodes, insects and fish

As mentioned before, nematodes and other invertebrates are more likely to utilize histone modifications and small RNAs as carriers of epigenetic information over DNA methylation. The epigenetic system in Caenorhabditis elegans has been described as highly efficient and includes RNA interference mediated by family of Argonaute proteins [91]. DNA methylation in C. elegans occurs mainly at adenine N6 (6mA) rather than 5mC in most other organisms [92]. As such, this organism has been used for studies of transgenerational epigenetic mechanisms using siRNA [93; 54; 94; 55] and histone modifications [76; 54].

Similarly, epigenetic inheritance in Drosophila melanogaster is mainly studied focusing on chromatin modifications, which has shown that intergenerational reprogramming in response to dietary manipulation modified the chromatin state and transcription in offspring of high-sugar fed fathers. Chromatin-dependent transcriptional derepression in the sperm represented an epigenetic signature predictive of obesity susceptibility were also found in murine and human obesity samples, suggesting that a congruent mechanism exists in mammals [50].

Vertebrate model, zebrafish Danio rerio shares basic DNA methylation enzymes such as DNMT1 with mammals [95; 96], however, the mechanism of gamete hypomethylation is strikingly different. The sperm methylome in zebrafish is inherited without significant changes throughout early development. The oocyte carries initially stable methylome too, but undergoes extensive methylation pattern remodeling to reset its epigenetic state to that of the paternal genome. The similarity between the blastula stage methylome and the sperm methylome

suggests that the paternal methylome might be inherited through germ cell development to mature sperm and entire zebrafish life cycle [97; 98].

3.3.1.3. Epigenetic inheritance in rodents

Studies of epigenetic inheritance in inbred rodent strains propose a way of investigating complex effects environmental factors can have on the offspring health with parallel to highly outbred human populations. Most of the rodent studies supporting the epigenetic transmission of parental experiences to offspring have been restricted to F1 generation because of long generation time and complex development. These studies have demonstrated various effects in models of exposure to dietary challenges [49], chemicals, toxicants, endocrine disruptors [53] or social stress [99; 100] and many others. Specific effects of environmental challenges observed in rodents are described in the next section in further detail.

3.3.1.4. Epigenetic inheritance in humans

Due to long generation times, difficulties obtaining human data and general outbred nature of human populations, it is problematic to pinpoint mechanisms of epigenetic inheritance in humans. A number of studies have, however, supported the concept that environmental and nutritional disturbances might result in non-genetic transmission of altered phenotypes across generations [44]. Focusing on the phenotypes leading to disease, the evidence of transmission of acquired traits in humans are described in further detail in the next section.

In regard to the epigenetic mechanisms in humans, it is important to mention imprinting disorders which stem from errors in gene expression, which is dependent on parent-of-origin specific manner. Genomically imprinted chromosomal regions and genes are expressed only from to maternal or paternal allele, but not both. Imprinting disorders alter their regulation, as imprinted loci contain several genes under coordinated epigenetic control. Disorders in imprinting affect growth, development and metabolism in offspring. Children with identical genotypes (deletion of 15q11-13) have contrasting phenotype of Angelman or Prader-Willi syndrome, depending on which parental copy of the region is present. In Angelman's syndrome, the 15q11-13 chromosomal region contains genes expressed from paternal alleles leading to

developmental delay and abnormal behavior with typical excessive laughter. Conversely, maternally expressed genes from 15q11-13 region in Prader-Willi syndrome contribute to excessive eating, hypogonadism and obsessive-compulsive disorder in affected children. Other imprinting disorders include Silver-Russel and Beckwith-Wiedemann syndrome which differ in parental-copy-expression of 11p15 chromosomal region and Temple and Kagami-Ogata syndrome linked to differences in imprinting of 14q32 region [101]. Another example is transient neonatal diabetes mellitus type 1 (TNDM1) which is associated with overexpression of maternally imprinted gene PLAGL1/ZAC located in 6q24 [102].

3.3.2. DOHAD – Developmental origins of health and disease

The field of biomedical science and public health known as the developmental origins of health and disease contains research of how adverse environmental factors during early human development influence the risk of later chronic disease [103]. The processes of developmental plasticity in early life with potential adverse consequences later are mainly physiological [104]. Without extensive knowledge on the subject, researchers often used a phrase "programming of disease/function" which stemmed from genetic program of development. The use of the term "programming" is in this manner ultimately interchangeable with "conditioning" as the environment conditions an individual to respond physiologically to later environmental challenges in a specific way [104]; however, I will use this term throughout the thesis in mere descriptive way of the effects of studied nutritional challenges.

The biological phenomena that may connect intrauterine nutritional experiences and subsequent health outcomes of the offspring was also described by the term "metabolic imprinting". This term was based on the historical precedent of Konrad Lorenz, who used the term imprinting to describe powerful setting of animal behaviors that resulted from an experience early in life. Metabolic programming/imprinting/conditioning or developmental plasticity are all terms that are intended to represent adaptive responses of the organism to specific conditions in early life that are characterized by

1. a susceptibility limited to a critical window of early development,
2. a persistent effect lasting up to adulthood,
3. a specific and measurable outcome, which may differ among individuals,

4. an existence of a threshold or a specific dose relation between the exposure and outcome [105].

If we were to analyze the practicality of such adaptive processes, we must include evolutionary biology in the thought. The physiological processes had to be selected during evolution as they confer an adaptive advantage in terms of survival to the age of reproductive ability. The risk of chronic diseases rapidly increases in the post-reproductive period due to accumulative effects of consequences of lifestyle, behavior and environment. They represent a mismatch between conditions of individual's development or conditions of their evolutionary ancestry and current challenges with an element of evolutionary novelty. As a result, the physiological phenotype of the individual does not match their current environment, which can nevertheless occur at any time of their life. It is important to mention that many of the mismatched conditions have arisen from climate change, pollution, Westernization and socioeconomic progress [104].

3.3.2.1. Historical background of DOHAD

During World War II, the German occupation of the western region of the Netherlands embargoed food supplies from October 1944 until liberation in May 1945. People of all social classes living in the area, including pregnant women, received as little as 400-800 calories a day. The Dutch Hunger Winter or the Dutch Winter Famine 1944-1945 was later described as a tragic human experiment of the effects of intrauterine deprivation on subsequent adult health and provided major findings and insights crucial for establishing the field of DOHaD [106].

After 30 years, the first studies regarding the famine period in the Netherlands started to appear, ultimately leading to one of the most extensive research of the famine cohorts. Depending on the stage of gestation women affected by famine were in, the consequences on their babies' health varied. Women who gave birth during the famine and were exposed to undernutrition in the late gestation not only showed reduced body weight postpartum, but also delivered babies with lower birthweights which displayed decreased glucose tolerance at the age of 50 compared to individuals born the year before or after famine [107]. Individuals of this group were also less likely to be obese in adulthood, compared to individuals exposed to famine in the first or second trimester *in utero* [108]. The exposure to famine in the second trimester was connected to a higher prevalence of glucose intolerance, microalbuminuria and obstructive

airways disease in affected people. Mid gestation is characteristic for rapid growth of the bronchial tree and therefore the findings support the hypothesis that fetal undernutrition in this critical period of airway development permanently affects their structure and physiology [109]. Similarly, it is possible that undernutrition in this period prevents formation of sufficient glomeruli and affect renal function in adulthood [109]. Exposure to famine during early gestation was associated with an increase in prevalence of coronary heart disease later in life [110]. Individuals that were undernourished in utero during the first trimester of their mothers' pregnancy were also more likely to suffer from glucose tolerance impairments, altered blood coagulation, atherogenic lipid profile and obesity [109; 106]. These effects were independent of reduced birthweight, as the delivered babies exposed to undernutrition in early gestation were normal-sized [108; 111]. Early gestation is characteristic by the formation and growth of placenta, which provides the fetus with oxygen and nutrients, and is also influenced by food restriction or excess [112], therefore inadequate placental factors should be considered in this research.

In 1962, Neel proposed a hypothesis that suggested that the high incidence of type 2 diabetes in Pima native American people (New Mexico and Arizona) results from the existence of diabetogenic genes [113; 112]. These genes should aid in survival of the individual in conditions with scarce nutritional resources but are detrimental to survival in conditions of nutritional excess [113], such as westernized diet. The enhanced capacity to store fat when nutritional resources are scarce also places the individual at risk of insulin resistance and type 2 diabetes [113; 105]. It appeared to be a failure of natural selection to eliminate a lethal condition that was genetically determined [113]. However, in 1992, Hales and Barker have come up with a "thrifty phenotype hypothesis" which provided an alternative and suggested a role of inadequate early nutrition which results in impaired development of the endocrine pancreas and therefore increased susceptibility to the development of type 2 diabetes [114]. When fetal environment is inadequate, the adaptive response optimizes the growth of key body organs to the expense of others and leads to altered postnatal metabolism. These permanent metabolic adaptations maximize the chances of postnatal survival under similarly poor conditions, however when the nutrition becomes more abundant than those during the prenatal environment, the alterations in the metabolism serve as basis for metabolic diseases [105]. In connection to metabolic adaptations, cell numbers and functional capacities of critical organs are reduced. Pancreatic cells, reduced number of nephrons in kidneys and heart muscle cells are those markedly affected. In particular, Barker and colleagues associated lower birth weight

and weight at one year of age with an increased risk of death from cardiovascular disease and stroke in a cohort of men and women born in Hertfordshire, England between 1911 and 1930 [115; 116].

These findings were further supported by other studies, which linked lower birthweights with health complications in adulthood. The study of 15 000 Swedish men and women revealed increased death rates from ischemic heart disease in individuals in the lower quartiles of birth weight compared to those in the highest quartile [117]. Another study in Sweden reported that male army men who had been small at birth had a significantly higher risk of increased diastolic blood pressure [118]. A different study of cohorts from Preston, UK and Adelaide, Australia was not only looking at birth weight but also placental weight and reported that blood pressure increase was connected to decrease in birth weight and increase in placental weight [119; 120]. Individuals exposed to war-time during early and fetal development had an increased risk of hypertension following the civil war in Nigeria (1967 – 1970) [121]. Another study showed an association between hypertension following the Great Famine (1959 – 1961) in China during fetal development [122], however a different study did not [123].

3.3.2.2. Paternal effects on offspring metabolism

When investigating the paternal effects on offspring's phenotype, it is natural to focus on epigenetic inheritance. As males don't directly interact with the development of their offspring, the studies often target aspects of sperm biology. Sperm motility and even composition of seminal fluid are however also influenced by paternal conditions and can potentially alter offspring phenotype, nevertheless it is not clearly understood what the primary mechanisms are [45]. The view of paternal inheritance is generally limited to epigenetic mechanisms, but can be challenged by interesting findings from recent studies.

Paternal ancestral influence over offspring phenotype can be studied either from the point of ancestral genotype or environment. The effect of ancestral genotype was demonstrated by study of Nelson et al. which showed that genetically identical daughters of males differing only in their Y chromosome (which is not inherited by daughters) showed differences in lipid levels, bone density and even anxiety-related behaviors [124]. Effects of the environment never experienced by offspring are also able to influence their phenotype and are narrowed down to stress and nutrient availability for the purpose of this review [45].

3.3.2.2.1. Paternal stress

Stressful environments have many effects on future generations. Chronic variable stress during puberty or in adulthood of male mice was shown to induce reduced HPA-axis stress responsivity in the offspring. The paraventricular nucleus (PVN) and bed nucleus of stria terminalis (BNST), the regions of brain that regulate stress, displayed global changes in transcription patterns, including increased expression of glucocorticoid-responsive genes in the PVN of this offspring. The epigenetic mechanism behind the germ cell transmission seems to be specific sperm miRNAs, as nine of them were found to be significantly increased in paternal stress groups [125]. Sperm epigenome plays a potential role in embryogenesis and is likely affecting the developmental processes of brain function [126]. The role of sperm miRNAs in the transmission of paternal life experience has been shown in a study that performed a microinjection of the same nine micro RNAs into a single-cell zygote implanted into surrogate dams. The examination of HPA stress axis sensitivity in adulthood demonstrated that these animals had the same stress-induced dysregulated phenotype [127], previously reported in males directly subjected to chronic stress.

Perception of male's compatibility has also been found to impact maternal care and, therefore, indirectly affect the offspring. Painting the head of male Gouldian finch different colors was reported to alter female's investment in their offspring, in particular egg size, number and gender [128].

3.3.2.2.2. Effects of paternal nutrition

Transfer of family traditions regarding food from generation of ancestors to the generation of progeny is oftentimes important from both cultural and health perspective. Paternal ancestors in particular can have a distinct impact on the metabolism of their grandchildren. An interesting study on food availability in human populations revealed sex-specific transmission of cardiovascular disease and obesity risk to subsequent generations. The focus of this study was poor ancestral nutrition till early adulthood and how this period influenced the disease risk in their grandchildren. Kaati et al. found that the grandson's relative risk for disease was connected to the diet of his paternal grandfather, but not the paternal grandmother. On the contrary, the granddaughter's risk is related only to her grandmothers' diet. The window of time in which undernutrition occurred played a significant role in the

outcome of the grandchildren's phenotypes. If the grandparent had inadequate access to nutrition in early adulthood (~ 19 years old), the grandchild's mortality risk was reported to be increased, but in early adolescence (~ 10 years old) the poor nutrition of the grandparent was linked to a decrease in disease risk in grandchildren [129; 130]. Nonetheless, these types of studies are rather rare, as it is far more difficult to dissect the parental contributions that lead to F2 offspring phenotype outcomes in humans.

Alterations in glucose metabolism are common effects of parental nutritional challenges. Male mice, who were subjected to 24 h fast before mating have sired male offspring with decreased serum glucose levels [131]. A low-protein diet fed to male mice all their life from weaning to mating has also influenced their offspring's metabolism. Carone et al. showed that a low protein diet of fathers is linked to decreased levels of cholesterol esters and altered expression of cholesterol biosynthesis genes in the liver of offspring [49]. On the other hand, high-fat diet feeding in male rats influenced the metabolism of their daughters, which were reported to have decreased glucose tolerance and the number of cells in pancreatic islets [132]. Streptozocin-induced hyperglycemia, without additional metabolic burdens of obesity, was shown to induce more weight gain in rat offspring of hyperglycemic fathers. Compared to euglycemic father sired offspring, they also displayed hyperphagia and impaired brown adipose tissue thermogenesis, which may underlie the observed obesity phenotype. The long-term impact of paternal hyperglycemia on dysregulation of energy homeostasis was also supported by the presence of impaired hypothalamic leptin signaling in affected offspring [133].

An interesting study from Wei et al. demonstrated, that even prediabetes in the father has the potential to affect the offspring's metabolism. The methylome analysis of sperm of prediabetic mice revealed numerous differentially methylated genes, including genes partially resisting postfertilization methylation, which supported the notion of intergenerational transmission of cytosine methylation at a substantial portion of the genome [134]. Furthermore, increased fat accumulation linked to a prediabetic increase in serum glucose, insulin and leptin can consequently increase testicular temperature, which may affect the DNA reprogramming of the gamete [135]. Paternal diet-induced obesity was also shown to negatively influence preimplantation embryo development and reduced implantation rate in mice [136], which was also suggested by human studies [137; 138] [139; 140].

Independent from the cause, being small for gestational age was found to propose comparable risk for transmission to the next generation in fathers as well as in mothers. This

common fetal growth indicator is typically defined as birthweight below the 10th centile of the birthweight distribution according to gestational age [141] and presents the highest risk for the offspring to be small for gestational age, if both mother and father were small for gestational age. Given several maternal mechanisms, such as maternal constraint limiting fetal growth, it is important to note that paternal inheritance pattern of being small for gestation age provides a stronger argument for genetic contribution [142].

3.3.2.3. Maternal effects on offspring metabolism

Maternal environment is, without a doubt, crucial for the health of the developing fetus and remained the main focus of studies investigating diseases later in life of the offspring. The period of conception to birth is a time of cellular replication, differentiation and maturation of organs [143], which are all sensitive to alterations in nutrient availability or the impact of environmental chemicals. The inheritance of disease risk from maternal side seems to be multifactorial with metabolic, epigenetic and mitochondrial contributing factors.

3.3.2.3.1. Placental factors and maternal constraint

With the historical focus on low birth weight, the implications of studies showing long-term effects on disease risk associated with birth weights within normal range [144; 115; 145] were often ignored. In all pregnancies, we recognize the processes of maternal constraint, which are highly relevant. They involve a set of uteroplacental mechanisms by which the fetal growth is restricted in order to permit successful passage through the pelvic canal at delivery. In this sense, the fetal growth is practically restricted from reaching its genetic potential [146-148] as it presents an incidental price humans have paid for the evolutionary advantage of upright posture and bipedal movement.

In connection to maternal constraint, studies on animal models showed that induction of intrauterine growth restriction (IUGR) by ligation of uterine artery elicits a distinct response in delivered offspring. In adulthood, IUGR rats (bilateral uterine artery ligation at day 18 of gestation) develop diabetes with progressive dysfunction in insulin secretion and action [149; 150]. Mothers, however, mostly balance the effects of suboptimal nutrition and fetal growth is

adversely impacted only in the face of severe maternal malnutrition. IUGR rarely appears in humans as a result of malnutrition and is often a consequence of uteroplacental insufficiency caused by maternal smoking, anemia, pre-eclampsia or hypertension. Uteroplacental insufficiency reduces levels of glucose, insulin, insulin-like growth factor 1 (IGF-I), amino acids, fatty acids and oxygen being available to the fetus [149]. Decreased availability of these essentials not only alter metabolic pathways and signaling systems, but can also reprogram the mitochondrial function [151; 152]. Reduction in energy supply and oxygen activates the mitochondria in the fetus [153; 154], which can lead to increased production of reactive oxygen species (ROS) and to oxidative stress which can have detrimental effects on cells with high energy requirement, such as pancreatic beta cells, myocytes, hepatocytes and placenta [155-160]. Pancreatic islets of IUGR rats exhibit declines in ATP production and activities of complexes I and III of the electron transport chain [159]. Selak et al. observed that in IUGR muscle, the energy-dependent GLUT4 recruitment to the cell surface is compromised by impaired ATP synthesis and contributes to insulin resistance and hyperglycemia [157]. Hepatic mitochondria from IUGR pups prior to the onset of diabetes contained decreased oxidation rates of pyruvate, glutamate and alpha-ketoglutarate which predisposed rats to increased hepatic glucose production by suppression of pyruvate oxidation and increased gluconeogenesis [156]. Impaired mitochondrial function is not the only effect of IUGR, as studies also showed changes of epigenetic modifications in specific target genes, in particular promoters of key developmental transcription factors. IUGR induces alteration of the expression of the homeodomain-containing transcription factor PDX1 via epigenetic modifications [161]. PDX1 plays a critical role in the early development of exocrine and endocrine pancreas and in beta cell function. Levels of *Pdx1* mRNA were found to be more than 50% lower in IUGR rats (*in utero*) as early as 24 h after the induction of IUGR and altered *Pdx1* expression persists after birth. *Pdx1* expression is also decreased in human pancreatic islets in individuals with type 2 diabetes [162].

3.3.2.3.2. Role of maternal care and stress

Parental stress and trauma have been also shown to induce emotional and behavioral disorders in offspring over several generations. In general, the behavioral adaptation helps to adjust to changing environment, but when the conditions change too rapidly, it can cause a mismatch with the adapted behavior [39]. A discrepancy between individual's response and

surrounding conditions can lead to pathological behaviors and can increase the predisposition to disease [38]. Since prenatal period, early childhood and adolescence are critical temporal windows for the influence of environmental conditions in mammals, it is important to note that during these developmental phases, the brain experiences extensive growth [163] and remodeling [164] and is therefore very sensitive to external conditions [43].

Reduced maternal care as well as adverse parental experiences during early life have been reported to disarrange neurodevelopmental processes and represent a major risk factor for the development of mood disorders. F1 female rat offspring that received a higher quality maternal care such as increased grooming, licking and arch-back nursing from mothers shown subtle hypothalamic-pituitary-adrenal (HPA)-axis responses to stress than those not cared for so intensively [165]. These females also showed decreased fearfulness which suggests that quality of maternal care influences behavioral responses and causes adaptive behaviors in F1 female offspring. Weaver et al. have identified DNA methylation and histone modification changes at the nuclear receptor subfamily 3, group C, member 1 (*Nr3c1*) locus in the hippocampus of the nurtured F1 female pups during the first week of postnatal life. These changes were correlated with changes in *Nr3c1*-encoded glucocorticoid receptor expression, however, they are not likely transmitted through gametes [165; 44]. Nurturing behavior represents a learned trait and is probably acquired through epigenetic regulation in genes such as oestrogen receptor alfa1b in the medial preoptic area of the brain [166].

Social stress exposures during early development induce emotional alterations in the progeny, particularly chronic and unpredictable maternal separation [99; 125; 100]. This specific type of stress in postnatal days 1-14 led to altered DNA methylation of the methylated CpG binding protein 2 (*Mecp2*), cannabinoid receptor 2 (*Cnr2*) and corticotropin release factor receptor 2 (*Crfr2*) genes in adult sperm of stressed males. Subjecting mice to this type of stress-induced depressive behaviors and some of the behavioral alterations were transmitted F2 female and F3 male offspring through stressed F1 males [99]. On top of depressive behaviors, unpredictable maternal separation was shown to lead to social withdrawal, impaired social recognition and reduced risk assessment [99; 167; 168].

The effects of maternal care are also culturally inherited and the quality of maternal care is thus propagated over generations [45].

3.3.2.3.3. Chemicals and endocrine disruptors

A study involving exposure of female rats to endocrine disruptors vinclozolin and methoxychlor at a specific time has highlighted a phenomenon of broad effects persistence in future generations long after the environmental stressor had passed. Embryonic day 8-15 in rat is a critical time during gonadal sex determination and toxin exposure at this time resulted in decreased spermatogenic capacity and male infertility over at least four generations. This phenotype was associated with altered DNA methylation in genes of the male germline [53]. Epigenetic inheritance of abnormal phenotypes that were induced by dioxin, pesticides, insect repellents and plastic-derived endocrine disruptors [169-172] indicate that environmental chemicals are able to create long-lasting changes over generations.

Bisphenol A (BPA) is a man-made synthetic chemical commonly used in packaging materials, medical devices and even dental sealants. The range of exposure to BPA spans from ingestion and inhalation to dermal contact, which yields measurable levels of BPA in urine, for example [173-175]. BPA is what we recognize as endocrine disrupting chemical (EDC), which increases the risk of endocrine-related disorders in humans and in animals, and precisely because of its ubiquitous presence in the environment received a lot of attention in research. Evidence suggests that endocrine disruptors not only affect the health of directly exposed populations, but also their sired offspring. Studies of Bansal et al. reported that early-life exposure to BPA in mice was associated with sex- and dose-specific effects on metabolism, including reduction in beta cell mass and increased glucose-stimulated insulin secretion spanning 3 generations [176-178]. Metabolic dysfunction was also linked to phthalates, organotins, polychlorinated biphenyls, dioxins, nitrogen oxides, polyaromatic hydrocarbons and parabens which are chemicals we come in contact with daily.

3.3.2.3.4. Maternal nutrition

As mentioned previously, epigenetic changes are the main focus of studies considering the developmental origins of adult disease and partly explain how a time-limited stimulus in early life can have long-lasting consequences [143]. The epigenetic modifications are directly influenced by the nutritional state of the organism and rely upon substrates derived from one-carbon metabolism such as S-adenosyl methionine (SAM), acetyl CoA, alpha ketoglutarate and nicotinamide adenine dinucleotide (NAD+) [179]. Foods containing methionine, serine, folate,

biotin and choline provide a dietary source of methyl groups that are transferred to DNA and histones through SAM [180]. A classic example of how parental nutrition influences DNA methylation in offspring was presented by studies of *agouti viable yellow* (A^{vy}) or *axin fused* ($Axin^{Fu}$) mice. Since A^{vy} mice carry an intracisternal A particle (IAP) retrotransposon upstream of the Agouti gene, these animals exhibit a wide range of coat colors due to differences in DNA methylation at the IAP element [181-183]. The murine agouti gene encodes a paracrine signaling molecule that signals follicular melanocytes to switch from the production of black eumelanin to yellow phaeomelanin. Transcription of agouti gene differs in initiation that starts from different promoters in each allele (wild type versus nonagouti caused by loss-of-function mutation). A^{vy} allele resulted from the insertion of IAP retrotransposon into the 5' end of wild type allele and CpG methylation in this region correlates with agouti expression. Dietary methyl supplementation in pregnancy of A^{vy} female mice shifts the coat color distribution of their offspring, with a larger percentage of the offspring having a wild-type color coat [184] and prevents transgenerational amplification of obesity [185]. A study performed on humans revealed the correlation of combination of high folate and low vitamin B12 concentrations in maternal diet and increased risk of insulin resistance and obesity in children [186]. In addition, another human study observed that folate supplementation in pregnancy reduced the risk of metabolic syndrome in children [187].

In addition to methyl donors, other micronutrients are essential for proper development and health maintenance in mammals. Magnesium intake below the recommended dose has been associated with chronic inflammation, osteoporosis and components of metabolic syndrome such as obesity, diabetes, atherosclerosis and hypertension [188]. Magnesium deficiency has also been associated with insulin resistance, which improved in diabetic patients following oral supplementation. Even non-diabetic patients benefited from magnesium supplementation by improvement of insulin sensitivity [189; 180]. Maternal and postnatal levels of magnesium are also important for programming of body adiposity and insulin secretion in rat offspring [190]. Moreover, another study observed that maternal low chromium diet increased body weight and adiposity in rat offspring [191].

Caloric restriction during pregnancy and lactation also negatively impacts offspring health. Restriction of maternal food intake to just 30% of ad libitum leads to hyperphagia and an increase in adiposity in the offspring [192]. When provided with a high-fat diet programmed animals developed obesity, and in general, obese animals decrease their locomotor activity

[193]. Researchers have therefore suggested that fetal undernutrition programs a "couch-potato" syndrome (high food intake and low energy expenditure through physical activity [193]. Another study showed that 50% restriction in food intake during pregnancy and lactation of rats resulted in decreased histone acetylation and increased H3K9 methylation in *Glut4* promoter in skeletal muscle of the growth-restricted offspring. This creates a metabolic knockdown of *glut4*, which is an important regulator of peripheral glucose transport and insulin resistance [194]. Similarly, protein-restricted rat dams gave birth to offspring with hypermethylated P2 promoter of *Hnf4α*, a pancreatic transcription factor [195] important for the development and function of beta cells. In connection with repressive histone modification at the P2 promoter of *Hnf4α* the expression of the transcription factor was significantly reduced. Protein restriction during pregnancy and lactation of rat dams also reduced mitochondrial DNA content in liver, pancreas and skeletal muscle of offspring [196] which was similar to effects in piglets [197]. Moreover, protein restriction in this period promoted hypertension [198] and renal dysfunction [199] in the offspring.

Undernutrition of F0 mothers increases risk for the development of obesity and diabetes in her F1 offspring. F1 females therefore stand as high-risk and when they themselves become pregnant, the metabolic stress of pregnancy can possibly result in hyperglycemia and gestational diabetes. Studies have shown that maternal diabetes can contribute to hyperinsulinemia in the fetus and increased risk for the development of obesity and diabetes in offspring [200], but also F2 offspring. In this case, the transmission of phenotypes occurs through the maternal lineage. Caloric restriction during the last week of gestation in pregnant F0 mice was linked to impairment of fetal growth, 15-20% reduction in birth weight and subsequently to the development of obesity and glucose tolerance in F1 offspring [201; 202]. These effects resulted from impaired glucose-stimulated insulin secretion [201] and were transmitted to the F2 generation as well [203]. The impairment of insulin secretion was also linked to altered activity of ATP-sensitive potassium channel in islets of F2 offspring, which is part of the pathway regulating insulin secretion from beta cells. The expression of *Sur1* (sulfonylurea receptor), a gene coding ATP-binding cassette protein which is a part of KATP channel, was reduced by 30% in F1 and F2 offspring pancreatic islets. The results indicated that dysregulation of *Sur1* gene expression and function can be transmitted to F2 through both parental lines. Jimenez-Chillaron et al. have further reported that in mice, reduced birth weight was transmitted from F1 to F2 generation through the paternal, but not the maternal line and that impaired glucose tolerance and beta-cell dysfunction was passed onto the F2 offspring from

both parental sides. Interestingly, F2 offspring whose parents were both undernourished *in utero*, developed insulin resistance and increased adiposity through maternal, but not the paternal lineage [203]. The phenotypes in F2 generation were observable despite the *ad libitum* feeding during pregnancy of F1 mothers, which points to long-lasting effects of undernutrition of F2 generation's grandmothers (F0 mothers).

The effects of maternal undernutrition on offspring's health are still a relevant research topic, as socioeconomic status and access to nutritional resources are lower in certain communities. However, lately communities became burdened with the opposite problem - recent shift in dietary habits, which is a part of westernization and globalization, steering food intake in the direction of overnutrition. Easy access to calorie-dense processed foods such as fast food, snacks and sweets are often combined with low prices and provide a bottom-line solution for low-income individuals. The high cost of healthy foods is one of the major factors preventing people from lower- and middle-income communities to make healthy choices. The relative cheapness of unhealthy calories ultimately leads to a high price paid in people's health. Lower income inequality is one of the most substantial determinants of poor health, commonly explained by unhealthy behaviors and lifestyle and notably cultural and social barriers [204]. The affordability of processed calorie-dense foods also poses as a risk factor for obesity and obesity-related issues in upper-income countries and represents a pressing worldwide problem.

The developmental overnutrition hypothesis proposes, that greater maternal adiposity during pregnancy increases the offspring's adiposity later in life. In particular, high maternal glucose, free fatty acids and amino acids plasma concentrations impact appetite control, energy metabolism and neuroendocrine system in the developing fetus. Such changes in fetal physiology can be permanent and passed onto the next generations. Mothers who are obese at the time of their pregnancy and breastfeeding can therefore perpetuate obesity in their children, which can accelerate the obesity epidemic independently of genetic or environmental factors [205-208]. Maternal BMI was found to be positively associated with fat mass of the offspring assessed at age 9 or 11 [208; 209] as a result of developmental overnutrition. Another study revealed that parental adiposity was positively associated also with fasting insulin concentration and HOMA-IR in 9.5 years old children [210]. This hypothesis generally proposes stronger maternal effects, which are in their nature more direct than paternal, however, paternal overnutrition was also associated with offspring's obesity and glucose intolerance, as described previously. Contemporary view suggests comparable contributing relative effects of both

paternal and maternal adiposity on offspring health. Even though maternal obesity has long-term impacts on offspring's health, interesting study of Kral et al. showed that significant weight loss improves obesity-related parameters in children. The study involved siblings who were born before or after their obese mothers (BMI 48 kg/m^2) underwent a bariatric surgery (biliopancreatic bypass) and lost a substantial amount of weight. The prevalence of obesity in children born after their mother lost weight decreased by 52% and was comparable to prevalence of overweight and obesity in general population [211]. It is necessary to mention that parameters like BMI or weight, that are often used in scientific studies as descriptive values of adiposity, are not in fact always in correlation with it. This phenomenon is portrayed by the existence of normal-weight obese individuals with adequate body mass index (18.5 – 24.9 km/m^2) with excess body fat, who have an increased risk of developing noncommunicable chronic diseases despite the absence of consensual obesity. Body composition should be therefore assessed using additional anthropometric parameters such as dual-energy X-ray absorptiometry for measurement of body fat percentage, measurement of waist circumference, calculation of waist-to-hip ratio [212] and skinfold thickness (e. g. subscapular, triceps, biceps, suprailiac).

Maternal obesity causes changes in the amount of nutrients and composition of metabolites that pass through the placenta [213] and is associated with systemic inflammation [214]. Inflammation and insulin resistance lead to increased adipose tissue lipolysis and increase the free fatty acid levels in the fetus, which was reported by a study of chronic maternal high-fat diet feeding, when mothers received 35% calories from fat in their diet. Nonhuman primate offspring of mothers fed high-fat diet had elevated hepatic expression of gluconeogenic enzymes, 3-fold increase in liver triglycerides and showed increased hepatic oxidative stress which leads to the development of nonalcoholic fatty liver disease (NAFLD) [215]. Systemic inflammation connected to high maternal BMI was characterized by an increase in pro-inflammatory cytokines such as monocyte chemo-attractant protein 1 (MCP-1) and tumor necrosis factor alpha (TNFα), which were also found in placenta after high-fat diet [214]. Excess fetal lipid exposure in combination with inflammation can have serious impact on the development of brain, liver, adipose tissue, skeletal muscle and pancreas [216].

Study in rats revealed that maternal high fat diet affects epigenetic alterations in the offspring's proopiomelanocortin (POMC) gene in hypothalamus [217]. Proopiomelanocortin is a pro-hormone which many active peptides and hormones are derived from, such as β-

endorphin, adrenocorticotropic hormone and melanocyte stimulating hormone. POMC regulates food intake and energy balance through a complex network of neural pathways – melanocortin system. POMC neurons have been found to integrate signals such as leptin, glucose and insulin by inducing satiety and increasing energy expenditure [218]. Changes in offspring hypothalamus after maternal high fat diet alters expression of leptin receptor, POMC and neuropeptide Y (NPY) [219], which regulate eating behavior and food intake by their anorexigenic and orexigenic effects. Leptin and insulin stimulate anorexigenic POMC while inhibiting orexigenic neuropeptide Y. Dysregulation of hypothalamic circuits, such as functional resistance to insulin and leptin, subsequently leads to increased food intake and excessive weight gain. Maternal obesity and excessive energy intake were reported to alter DNA methylation in promoter regions of hypothalamic genes [219; 220]. High-fat diet in pregnancy also altered the methylation and gene expression of dopamine and opioid-related genes leading to changes in feeding behavior [221; 222] and moreover, resulted in hepatic hypermethylation and increased expression of *Mmp9* gene (matrix metallopeptidase 9) which triggered the development of metabolic syndrome in animal models [223; 224]. Dopamine and opioid reward circuitry may also by affected by maternal high fat diet which induces a preference for palatable foods rich in sucrose and fat [225]. Epigenetic modifications and various effects of maternal high-fat diet, such as decreased insulin sensitivity were also observed to persist across at least two generations of offspring [226].

Obesity contributes to the impairment of insulin signaling in the peripheral tissues, hyperglycemia and development of type 2 diabetes. Glucose is one of the main metabolic factors that epigenetically regulate gene expression, as it modulates the transcription of insulin gene by hyperacetylation of histone H4. The histone acetyltransferase p300 interacts with the beta cell specific transcription factor Pdx-1 and is recruited to the insulin promoter only at high concentrations of glucose [227; 228; 180]. Obesity, as well as increasing maternal age, parity (number of times that a woman has given birth) and common endocrine disorders such as polycystic ovary syndrome (PCOS) increase the risk for the development of gestational diabetes mellitus. Maternal gestational diabetes is also associated with risk for obesity and diabetes in the offspring [229; 230], due to materno-fetal transfer of excess glucose and other molecules during pregnancy. The developing fetus utilizes maternal glucose, which is transported through the placenta. Maternal insulin does not cross the placenta, and therefore the fetus has to balance the glucose load by increasing production of insulin on its own [231]. Excess glucose transport, such as in maternal hyperglycemia can contribute to neonatal adiposity and increased size at

birth, which increases the risk for neonatal complications such as shoulder dystocia and birth trauma [232]. Offspring born to mothers with gestational diabetes was also reported to have elevated triglyceride levels, higher markers for insulin resistance such as HOMA-IR and bigger waist circumference [233; 234]. Moreover, 20% of offspring born to mothers with gestational diabetes develop prediabetes and type 2 diabetes by age 22 [235-237].

Changes in human diets in the last decades, as well as common factors contributing to chronic diseases such as sedentary behavior and sleep disturbances revealed increased human susceptibility to the development of obesity and diabetes. In order to explain these observations, a recent model of disease risk proposed by Wells has expanded the thrifty phenotype hypothesis to a broader model called capacity-load model [238]. In this model, two factors are emphasized:

1. metabolic capacity or traits that promote the capacity for homeostasis, and
2. metabolic load or phenotypic traits that challenge homeostasis [239].

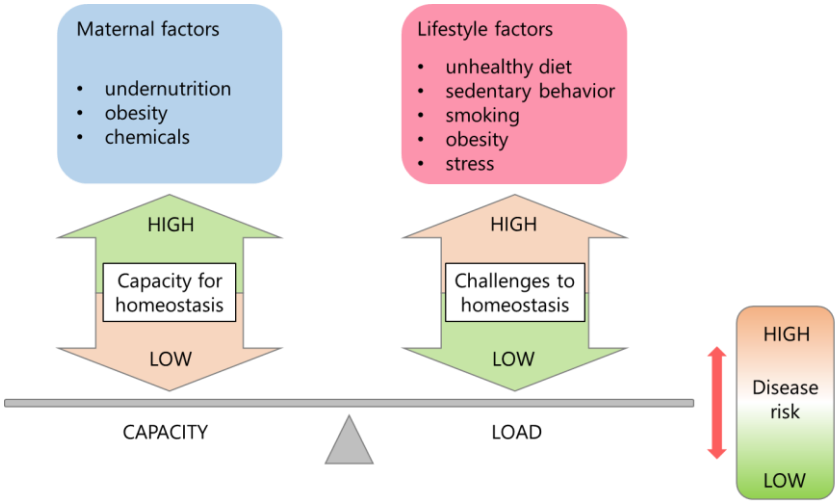


Fig. 1. Schematic representation of capacity – load model proposed by Wells.

In the discussion about diabetes, there are several key aspects of metabolic capacity, such as pancreatic function linked to insulin production and muscle mass, which is important in glucose clearance. Both of these are strongly influenced by *in utero* metabolic programming, as was reported from extensive research in the field of DOHaD. In relation to diabetes, load can be represented by increased adiposity, dietary glycemic load, sedentary lifestyle and psychosocial stress. Metabolic load can be in this sense composed of consequences of voluntary

actions (lifestyle) and involuntary environmental exposures. Dose-response associations in this model are interactive and therefore, diabetic risk increases directly with load and inversely with capacity, which was observed by studies: birthweight as a marker of capacity shows weak association with diabetes among humans with low load – healthy lifestyle, but a strong inverse association with diabetes risk in adults with high load represented by unhealthy lifestyle [239; 240; 238].

Greater carbohydrate intake during pregnancy was also a focus of studies trying to dissect the effects of maternal macronutrient intake imbalance. Studies in humans revealed a connection between higher maternal intake of fat and carbohydrates and higher neonatal adiposity [241; 242]. Conversely, higher maternal protein intake during the second and third trimester of pregnancy was associated with lower neonatal abdominal adiposity [243] and abdominal fat mass during adolescence [244]. In rats, high-sucrose diet fed during pregnancy was reported to alter glucose and insulin tolerance in weaned offspring and at 3 months of age in combination with increase in liver triacylglycerols [245]. Nonetheless, study of Sedova et al. reported that high sucrose diet feeding to rat dams did not induce predictive adaptive protection of glucose tolerance and insulin sensitivity. PD/Cub offspring programmed by high sucrose diet displayed increased insulin sensitivity of the skeletal muscle and high levels of adiponectin, which prevented glucose intolerance [246]. Sucrose is a disaccharide composed of molecules of glucose and fructose. As food industry proceeds to add sugar to various processed foods either in form of refined table sugar or high-fructose corn syrup, our consumption of fructose has increased dramatically. Fructose metabolism differs from glucose metabolism, as it does not induce insulin release and also requires different enzymes in the initial steps of metabolism. Fructose is mainly metabolized in liver, when it's oxidized to CO_2 and converted to lactate and glucose and leads to ATP depletion and uric acid production [247]. Fructose intake induces hypertension via numerous pathways including endothelial dysfunction, nitric oxide deficiency, activation of renin-angiotensin system and renal dysfunction, as fructose increases the reabsorption of salt and water in the kidneys and reportedly induces renal hypertrophy in rats [248-250]. Adult offspring of Sprague-Dawley rat dams fed fructose in pregnancy showed increase in blood pressure and hypertension at 12 weeks of age in number of studies [251-253]. Rat offspring of dams fed a diet with 60% fructose prior to conception and during pregnancy showed increased levels of serum insulin and free fatty acids, when they were exposed to fructose postnatally. Rat offspring only exposed to maternal high fructose diet exhibited dyslipidemia and increased hepatic lipid accumulation. Interestingly, in rats weaned to control

diet, these fructose-induced phenotypic changes were fully reversible by bitter melon (Momordica charantia) supplementation [250]. Bitter melon has been used as a folklore medicine for treatment of diabetes [254], showing that ethnobotanical knowledge is able to help prevent modern global health problems as well.

3.4. Animal models

Animal models, specifically mammals are used in scientific research due to anatomical and physiological similarities to humans. Scientific investigation, achieved often by applying various techniques and creating an impact on the organism, is by the status quo unethical in humans. Therefore, use of mammalian models for understanding of complex mechanisms or even assessment of therapies is crucial before human application. Not all results obtained from such studies can however be directly translated to human medicine. Through use of generations of laboratory rat we were able to investigate various effects of maternal dietary carbohydrates on their progeny, which is legally not possible to study in genetically diverse human population.

In our study, we used two inbred strains of rats with the intention of observing the impact metabolic programming possibly has depending on a particular genetic background.

3.4.1. Spontaneously hypertensive rat (SHR)

First strain used in the study was the spontaneously hypertensive rat (SHR/OlaIpcv, RGD ID 631848) as commonly used model of essential hypertension [255] was used because of its known metabolic abnormalities [256]. SHR is a polygenic model of hypertension which is reflective of human polygenetic hypertension risk. SHR was created from outbred albino Wistar strain and in process of selective breeding fixed hypertensive alleles in homozygous state. This genetic homogeneity allows the use of SHR as a model for cardiovascular diseases and metabolic syndrome as well [257].

3.4.2. SHR-Lx.PD5$^{PD\text{-}Zbtb16}$ congenic strain

The SHR-*Lx*.PD5$^{PD\text{-}Zbtb16}$ single congenic strain (SHR-Zbtb16 hereafter) carries the *Zbtb16* gene of polydactylous rat (PD/Cub, RGD ID 728161) origin on the SHR genomic background. The derivation of this strain was described previously [258-260]. This introgression of rat chromosome 8 region onto SHR background was related to metabolism alterations in the animals. These changes were related to the development of metabolic syndrome traits, such as decreased insulin sensitivity of the skeletal muscle and rise in postprandial triglycerides after dexamethasone administration [258]. The introduction of mutant *Zbtb16* allele causes preaxial polydactyly in hind legs/paws with less pronounced polydactyly-luxate syndrome as in PD/Cub [261].

The two strains therefore differ only in the variant *Zbtb16* gene. Both strains used in this study are highly inbred and maintained by brother x sister mating at the Institute of Biology and Medical Genetics, Prague.

3.5. *Plzf* gene – *Zbtb16*

The promyelocytic leukemia zinc finger (PLZF) protein was first identified in a patient with acute promyelocytic leukemia in 1993. Reciprocal chromosomal translocation t(11;17)(q23;q21) resulted in a fusion with RARA gene encoding the retinoid acid receptor α [262]. Promyelocytic leukemia zinc finger or zinc finger and BTB domain containing 16 (*Zbtb16*) is well conserved in mammals and expressed in most tissues. Human and mouse/rat *Zbtb16* show at least 96% identity. Zbtb16 is a nuclear phosphoprotein that acts as a transcription factor and contains nine Cys2-His2 zinc fingers that facilitate sequence-specific DNA binding to its target genes [263]. Apart from the RD2 repressor domain, the protein contains N-terminal BTB/POZ (bric-a-brac, tramtrack, brad complex/poxvirus zinc finger) multimerization/repression domain [264] that mediates transcriptional repression through recruitment of nuclear corepressors [265-267]. *Zbtb16* is a member of POZ and Krüppel zinc finger family of proteins that induce epigenetic changes – histone modifications and DNA methylation and regulate the chromatin state [268] [269].

Studies in mice revealed that *Zbtb16* acts as a transcriptional repressor of Hox genes in embryonic limb patterning and apoptosis [270-273]. Data from rat [274; 275] and human [276]

studies further supported the role of *Zbtb16* in limb development. The morphological aberration in polydactylous rat strain (PD/Cub) [261] – preaxial polydactyly was attributed to a specific *Zbtb16* mutation (Lx) comprising of 2,964 bp deletion in intron 2 of the gene [274]. Deletion removes several deeply conserved noncoding elements with suggested regulatory influence on *Zbtb16* expression. The expressivity and penetrance of the Lx mutation in significantly influenced by genetic background – in Brown Norway rats it also afflicts forelimbs and behaves as semidominant, in SHR it's recessive and restricted to hindlimbs [261; 277]. This restriction of the mutant phenotype to hindlimbs was also observed in mice homozygous for inactivated *Zbtb16* gene [270]. Study from Liska et al. has demonstrated, that Lx mutation in Zbtb16 influences limb development independently of sonic hedgehog (Shh), however corresponding changes in posterior *HoxD* gene expression suggest that they could act as effectors of polydactyly [274] in homozygous Lx/Lx rats and mice [270].

Zbtb16 is a pleiotropic factor involved in numerous processes and functions. The discovery of the protein in humans as a cause of retinoid acid-resistant acute promyelocytic leukemia commenced its relevance for cancer and immune system function [278], stem cell self-renewal [279] and hematopoiesis [280]. Zbtb16 is involved in maintenance of spermatogenesis [281], osteo- and chondrogenesis of mesenchymal stem cells [282; 283] [269]. Regulation of innate immunity is another important function of Zbtb16, as it is required in the interferon-mediated antiviral innate immune response *in vivo*. Zbtb16$^{-/-}$ mice are more prone to viral infections due to failed induction of interferon-stimulated genes and the impairment of IFN-induced activation of natural killer cells [284; 285].

The expression of *Zbtb16* has an important role in the adipogenesis in white adipose tissue. Mikkelsen et al. found that the overexpression of *Zbtb16* suppressed the adipogenesis in L1 cells and proposed that *Zbtb16* is an anti-adipogenic factor [286]. Zbtb16 overexpression in brown adipocytes increased fatty acid oxidation, glycolysis, increased number and activity of mitochondria and induced thermogenesis. Plaisier et al. also demonstrated that brown adipocytes also utilized more carbohydrates leading to a decrease in triacylglycerols content [287]. Furthermore, a targeted rat model SHR-Zbtb16$^{+/-}$ showed lower levels of cholesterol and triacylglycerols [288]in contrast to SHR-Zbtb16 rats, which were more likely to develop glucocorticoid-induced dyslipidemia compared to SHR controls [258].

Glucocorticoids are hormones synthesized and secreted by the adrenal cortex, which regulate many biological functions through binding to the glucocorticoid receptor.

Glucocorticoid receptor binds to glucocorticoid response elements in the promoters of gluconeogenic genes and triggers gluconeogenesis [289; 285]. On top of that, glucocorticoids regulate lipolysis [290] and various cardiovascular [291] and immunological processes [292]. Zbtb16 has a major response to glucocorticoids, which is cell type-specific depending on interaction of glucocorticoid receptor with cell-specific cofactors [285]. The elevated blood glucose levels induced by *Zbtb16* in mice were related to its positive regulation of gluconeogenesis but also by negative effects on the insulin signaling pathway by decreasing the phosphorylation of insulin receptor substrate 1 (IRS1), protein kinase B (Akt) and forkhead box class O1 (FoxO1) [285]. These findings support the hypothesis, that *Zbtb16* can play a role in development of metabolic syndrome symptoms and thus its specific functions are worth investigating further.

4. Material and methods

4.1. Ethical statement

All experiments were performed in agreement with the Animal Protection Law of the Czech Republic (311/1997) which is in compliance with the European Community Council recommendations for the use of laboratory animals 86/609/ECC and were approved by the Ethical Committee of the First Faculty of Medicine of the Charles University and by the Ministry of Education, Youth and Sports (protocol no. MSMT-14076/2015-14).

4.2. Animal housing

Animals were held under temperature and humidity-controlled conditions on 12 h/12 h light-dark cycle. At all times, the animals had free access to food and water. Animals were housed in groups up to 4 per cage, with an exception of pregnant females, which were housed individually in order to obtain proper measurement of consumed chow. After delivery, the pups were housed with their birthmother until weaning in 4 weeks of age, when they were housed in same sex groups up to 4 per cage.

4.3. Oral glucose tolerance tests and blood draw

Blood samples for metabolic and glycemic assessments were drawn after overnight fasting from the tail vein. The OGTT of females selected as mothers was performed at 16 weeks of age (adulthood) and on the 10^{th} day of pregnancy. The blood samples were obtained at intervals of 0, 30, 60, 120, and 180 min after intragastric glucose gavage to conscious rats (3 g/kg body weight, 30% aqueous solution; Ascensia Elite Blood Glucose Meter; Bayer HealthCare, Mishawaka, IN, USA; validated by the Institute of Clinical Biochemistry and Laboratory Diagnostics of the First Faculty of Medicine). The OGTT of adult offspring was performed at 6 months of age with the same protocol.

4.4. Experimental protocol

All animals were held under controlled conditions (temperature, humidity) with free access to food and water. F0 generation of rat dams of SHR (see 3.4.1.) and SHR-Zbtb16 (see 3.4.2.) strains came from standard breeding and were fed a standard diet till the age of 16 weeks when they entered the experimental protocol. Breeding protocol consisted of placing corresponding breeding males (SHR x SHR, SHR-Zbtb16 x SHR-Zbtb16) in the cage with females overnight. After removal of the male, the gravidity status of females was confirmed by the presence of sperm in native vaginal smears, which were evaluated by optical microscopy. Pregnant females were placed in cages individually and their body weight and the amount of consumed chow were measured weekly. The number of animals in experiments is summarized in Table 2 (see Příloha 1).

4.4.1. Study 1 – F0 mothers and F1 male offspring

F0 rat dams were fed standard diet till breeding with corresponding (SHRxSHR, SHR-Zbtb16 x SHR-Zbtb16) males fed standard diet. After mating, rat dams were placed in the cages individually and fed either standard diet (STD, ssniff Rat breeding V1324-000, ssniff Spezialdiäten GmbH, Soest, Germany) in control group or high-sucrose diet (HSD, proteins (19.6 cal%), fat (10.4 cal%), carbohydrates (sucrose, 70 cal%) prepared by Institute for Clinical and Experimental Medicine, Prague, Czech Republic) in experimental group (Fig. 3). The diets differed in the carbohydrate fraction only, with starch in STD vs. sucrose in HSD as a source of carbohydrates; otherwise they contained equal amounts of macro- and micronutrients. Each group was fed either STD (control) or HSD throughout pregnancy and lactation (HSD/HSD mothers). The litter size was restricted to 8 pups both in SHR and SHR-Zbtb16 offspring which were weaned after 28 days and fed standard diet till adulthood (Fig. 2).

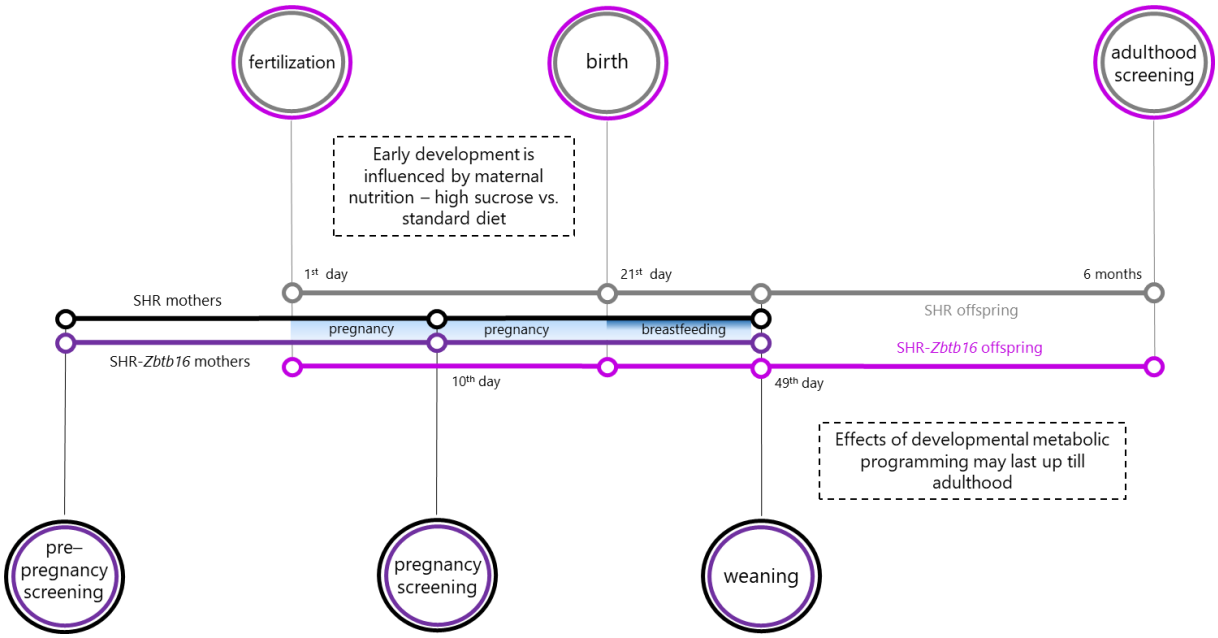


Fig. 2. Experiment timeline.

4.4.2. Study 2 – F1 male offspring subjected to HSD challenge in adulthood

The standard protocol was modified for selected group of male offspring which were subjected to nutritional challenge in order to assess their reaction to re-exposition to the same HSD which programmed their metabolism in early development. Male rats were fed HSD ad libitum for 14 days at 6 months of age and then sacrificed in the same manner as previous groups.

4.4.3. Study 3 – F1 female offspring and F2 male offspring

In order to test the hypothesis of programming effect transmission in multiple generations, we used F1 programmed females. Female offspring of F0 generation were fed STD till the age of 4 months and used as F1 mothers – mothers of F2 generation in the same breeding model as their mothers (see 4.4.1.).

F1 rat dams were weighed regularly and subjected to OGTT at 16 weeks of age to determine the differences in glucose tolerance after being metabolically programmed by HSD in prenatal and early postnatal development. Blood samples for metabolic and glycemic

assessments were drawn after overnight fasting from the tail vein, as described previously (see 4.3.). After the assessment of their adult glucose tolerance, the rat dams were mated with corresponding STD-fed males according to the breeding protocol (see 4.4.) and placed individually in cages. We assessed their weight gain, amount of chow consumed and again their glucose tolerance on the 10th day of pregnancy. Each group of F1 rat dams was fed standard diet whole 3 weeks of pregnancy and 4 weeks of lactation according to standard protocol. The litter size was restricted to 8 pups both in SHR and SHR-Zbtb16 offspring which were weaned after 28 days and fed STD till adult age of 6 months creating the F2 generation. At that time, SHR and SHR-Zbtb16 male offspring was subjected to OGTT, blood draw for metabolic and lipid profile assessment and sacrificed to determine the weights of heart, liver, kidneys, adrenals, interscapular brown fat, epididymal fat pad, retroperitoneal fat pads and skeletal muscle. The lipid profile was assessed using high performance liquid chromatography (HPLC) for determining triglyceride (TG) and cholesterol (C) concentrations in 20 lipoprotein fractions and the size of major classes of lipoprotein particles as described previously [293].

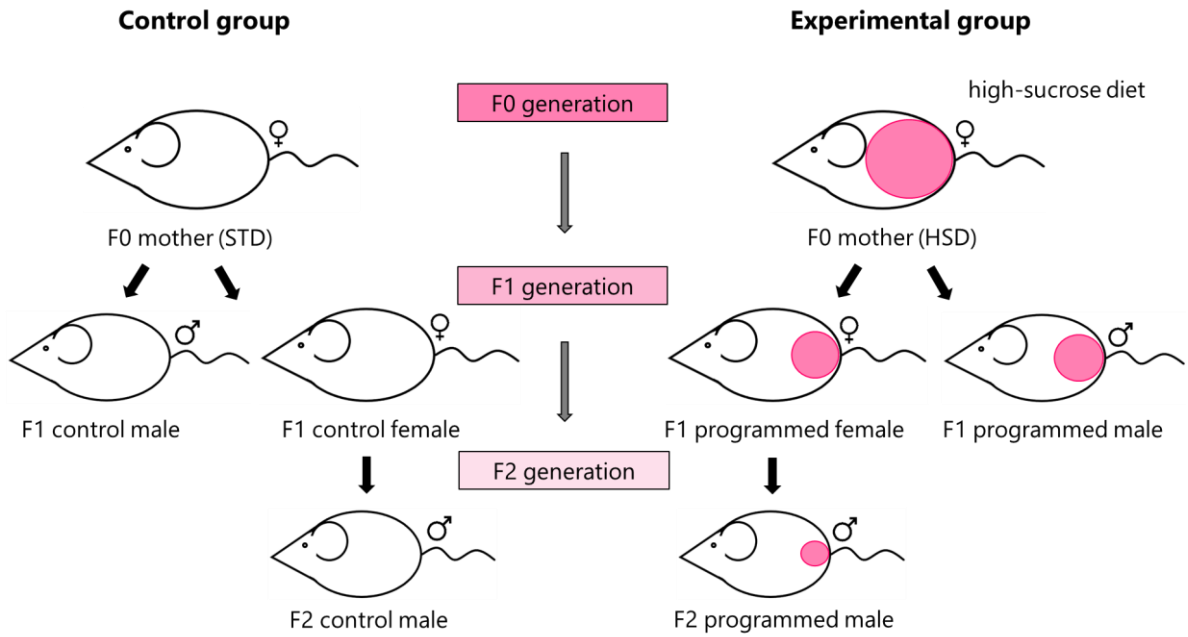


Fig. 3. Experimental design**.** Schematic display of three generations of rats involved in the experiment valid for both SHR and SHR-Zbtb16 strain. STD – standard diet, HSD – high-sucrose diet.

4.4.4. Unpublished studies

4.4.4.1. Pharmacological challenge – exposure to dexamethasone

Weaned male rats were fed STD until the age of 6 months, when they were randomly selected for the experimental exposure to dexamethasone. Adult animals were given dexamethasone (Dexamed, Medochemie) in drinking water (2.6 μg/ml) for 3 days as pharmacological challenge in order to assess their reaction based on metabolic programming. SHR-Zbtb16 male rats subjected to the challenge were not programmed, whereas SHR males were programmed with HSD in early development (F1 offspring of F0 mothers fed HSD/HSD).

4.4.4.2. SHR-Zbtb16 programmed with HSD in lactation period only

Selected group of F0 SHR-Zbtb16 females were fed HSD only in lactation period after delivery (STD/HSD females). Females were subjected to body weight measurements, glucose tolerance assessment and a blood draw after overnight fasting during pregnancy in the same manner as all the other mother groups. The resulting metabolic programming of the SHR-Zbtb16 F1 offspring was restricted to postnatal period. Male offspring of STD/HSD fed mothers were weaned on STD and the age of 6 months, selected animals were subjected to 14 days of HSD challenge. In order to compare the effects of various programming protocols, we compared not programmed males, STD/HSD programmed males and HSD/HSD programmed males which were all exposed to HSD for 14 days in adulthood. Female offspring of STD/HSD mothers were also weaned on STD and entered the breeding protocol (see 4.4.), their body weight and metabolic state was assessed and they were mated with not programmed SHR-Zbtb16 males. They were housed separately and fed STD during pregnancy and lactation and their male offspring was also weaned on STD. At the age of 6 months, the F2 STD/HSD offspring was assessed in the same manner as all the adult males in the experiment. F2 STD/HSD offspring were “grandmaternally” programmed by HSD – only in lactation period of their grandmothers (F0), as throughout the lives of their mothers (F1) and their own lives (F2) they were fed exclusively STD.

4.4.4.3. SHR programmed with maternal and grandmaternal HSD

Female offspring of F0 HSD/HSD programmed mothers were weaned on STD and used in the same breeding protocol (see 4.4) with not programmed SHR males to create F2 programmed generation. F1 HSD/HSD programmed females were programmed by maternal HSD during pregnancy and lactation and were divided to two groups. One group was fed STD throughout their own pregnancy and lactation and gave birth to F2 generation of "grandmaternally" programmed offspring (HSD/HSD STD/STD, see 4.4.3). The second group was fed HSD/HSD in the same period (HSD/HSD HSD/HSD) and became mothers of F2 programmed offspring, which were therefore programmed by both grandmaternal and maternal HSD.

4.4.5. Maternal (F0) metabolic assessments

The multiplex immunoassay system Bio-Plex® uses Luminex magnetic beads for the quantification of relevant protein targets of interest. In order to assess potential state of inflammation in rat dams of both strains, we used the multiplex assays for analysis 13 and 24 targets in little sample volume. Metabolic profile of female rat serum was assessed via 13-plex Milliplex®MAP Rat Metabolic Hormone Magnetic Bead Panel Kit using Bio-Plex® system (Bio-Rad), (Merck Millipore Corp., Billerica, MA, USA) for levels of amylin, C-peptide 2, ghrelin, GIP, GLP-1, glucagon, IL-6, insulin, leptin, MCP-1, PP. PYY and TNFα. Cytokine profile of rat dams was assessed via Bio-Plex Pro Rat Cytokine 24-Plex Panel (Bio-Rad Laboratories, Inc., Luminex Corporation) for levels of EPO, G-CSF, GM-CSF, GRO/KC, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12p70, IL-13, IL-17, IL-18, M-CSF, MCP-1, MIP-1α, MIP-3α, RANTES, TNF-α, VEGF using Bio-Plex® system (Bio-Rad). Multiplex immunoassays were performed at the Institute of Endocrinology, Prague, Czech Republic. The lipid profile was assessed using high performance liquid chromatography (HPLC) for determining triglyceride (TG) and cholesterol (C) concentrations in 20 lipoprotein fractions and the size of major classes of lipoprotein particles through Liposearch company, as described previously [293].

4.4.6. Offspring morphometric and metabolic assessments

Every born pup was weighted before litter size restriction to 8 pups, which were weaned after 28 days. The reason we restricted the litter size was to ensure that each pup would have access to the mother's nipple without competition with other littermates. Weaned rats were labeled and fed standard diet till the age of 6 months. We re-exposed some of the offspring to HSD challenge and dexamethasone treatment, creating more experimental subgroups, described in detail in the Results section. At 6 months of age male offspring of control and experimental groups were subjected to OGTT, blood draw for metabolic and lipid profile assessment and sacrificed to determine the weights of heart, liver, kidneys, adrenals, interscapular brown fat, epididymal fat pads, retroperitoneal fat pads and skeletal muscle (*musculus soleus*) from right hind leg. Liver, interscapular brown fat and epididymal adipose tissue were snap-frozen in liquid nitrogen in preparation for the transcriptome assessment.

4.4.7. Transcriptome assessment

4.4.7.1. RNA isolation

Total RNA was isolated from liver tissue (RNeasy Mini Kit, Qiagen), epididymal (visceral) and brown fat (RNeasy Lipid Tissue Mini Kit, Qiagen). For isolation of RNA from liver samples, we started with 30 mg of liver tissue and added 1 ml Buffer RLT and 10 µl beta-mercaptoethanol (β-ME) with 5.05 µl 0.5 % DX antifoaming agent. Next, we homogenized the samples in TissueLyser LT (Qiagen) for 10 minutes at 50 Hz with stainless steel beads. After homogenization we centrifuged the lysate at maximum speed for 3 min in room temperature and used the supernatant in the next step. We added 70% ethanol to the lysate 1:1, mixed well and transferred 700 µl of the mixture in RNeasy Mini spin column. We spun the columns for 30 sec at 8000 g (10 000 rpm), discarded the flow-through and washed the lysate with series of RW1 and RPE Buffers until we were able to eluate the RNA with 50 µl RNase-free water. RNA isolation from fatty tissues differed in first steps of lysate preparation. We homogenized 100 mg of fat tissue in 1 ml QIAzol Lysis Reagent with stainless steel beads using TissueLyser LT for 10 min at 50 Hz. After that we incubated the homogenate at room temperature for 5 min, added 200 µl chloroform and shook vigorously for 15 sec. After 2-3 min incubation at room temperature we centrifuged the sample at 12 000 g for 15 min at 4 ºC and transferred the aqueous phase to a new tube. We added 70% ethanol to the lysate 1:1 and proceeded the

protocol as described in liver tissue isolation. RNA from fat tissues was eluated with 30 µl RNase-free water. We measured the RNA concentration in spectrophotometer Nanophotometer P300 (Implen) and used it in later steps if the concentration hasn't exceeded 500 ng/µl. Due to extraction from RNA-rich tissues, we diluted the samples accordingly.

4.4.7.2. RNA integrity analysis

The quality and integrity of the total RNA was evaluated on Agilent 2100 Bioanalyzer system (Agilent, Palo Alto, CA). Agilent RNA kits contain chips and reagents designed for the analysis of total RNA. RNA chip contains an interconnected set of microchannels used for the separation of nucleic acid fragments based on their size as they are driven through it electrophoretically. We used Agilent RNA 6000 Nano Kit for the integrity analysis of total RNA from liver and fat tissues. In preparation, the RNA ladder was denatured for 2 min at 70 °C and immediately cooled on ice. Aliquots of denatured ladder were stored at -70 °C. RNA gel matrix was filtered through spin filter via centrifugation at 1500 g (4000 rpm) for 10 min at room temperature. To prepare gel-dye mix, one 65 µl aliquot of filtered gel and 1 µl RNA dye concentrate were vortexed together and spinned at 13 000 g (14 000 rpm) for 10 min at room temperature. After placing the chip on the priming station, we pipetted gel-dye mix in the marked wells and followed the manufacturer's instructions to evenly disperse the mix using the plunger of the priming station. At this stage, the chip was ready to be used for the electrophoresis. We pipetted 5 µl of RNA marker in all 12 sample and ladder wells. We used 1 µl of denatured sample as well as ladder in corresponding wells, horizontally vortexed the chip in IKA vortexer for 1 min at 2400 rpm and run it in Agilent 2100 Bioanalyzer instrument.

4.4.7.3. DNA microarray

To assess the transcriptome of liver and fat tissues we performed microarray using the Rat Gene 2.1 ST Array Strip in quadruplicate (per strain/programming). Using high-quality total RNA from isolation described above we followed the whole hybridization procedure using the Affymetrix GeneAtlas® system according to manufacturer's instructions. **First-strand cDNA synthesis** is a reverse transcription procedure in which the total RNA is primed with primers containing a T7 promoter sequence. It was completed using First-Strand Enzyme and

Buffer with 5 µl of total RNA and incubated in Biometra thermal cycler. The product of first-strand synthesis was a single-stranded cDNA with T7 promoter sequence at the 5' end and was used in the next step. After that we continued with **Second-Strand cDNA synthesis** using Second-Strand Enzyme and Buffer, similarly to the first step. In this procedure, single-stranded cDNA was converted to double-stranded cDNA, which acted as a template for *in vitro* transcription. The reaction used DNA polymerase and RNase H to simultaneously degrade the RNA and synthesize second-strand cDNA. In the process of **cRNA synthesis by in vitro transcription**, the antisense RNA (complementary RNA, cRNA) was synthesized and amplified by in vitro transcription (IVT) of the second-stranded cDNA template using T7 RNA polymerase. Similarly to previous steps, the cRNA was synthesized using IVT Enzyme and Buffer during 16 hour incubation in Biometra thermal cycler. The cRNA obtained in this step was then purified to remove enzymes, salts, inorganic phosphates and unincorporated nucleotides and to prepare the cRNA for second-cycle single-stranded cDNA synthesis. The **cRNA purification** was performed in U-bottom plate using Purification Beads and series of 80% ethanol washes on a specialized magnetic stand. We eluted the purified cRNA with 27 µl of Nuclease-free Water and assessed cRNA yield in spectrophotometer Nanophotometer P300. In **Second-Cycle Single-Stranded cDNA synthesis**, the sense-strand cDNA was synthesized by the reverse transcription of cRNA using Second-Cycle Primers. The sense-strand cDNA contained dUTP at fixed ratio relative to dTTP. We used 625 ng/µl cRNA (15 µg cRNA in volume of 24 µl) to enter the reaction, as well as Second-Cycle ss-cDNA Buffer, Enzyme and Primers, which was then incubated in Biometra thermal cycler. Next, we used RNase H to hydrolyze the cRNA template leaving single-stranded cDNA. To purify **Second-Cycle Single-Stranded cDNA** from enzymes, salts and unincorporated dNTPs and thus prepare the cDNA for fragmentation and labeling, we bound ss-cDNA to Purification Beads again and proceeded with the purification protocol as described above. At this point we used 30 µl of preheated (65 °C) of Nuclease-free Water to eluate the purified ss-cDNA and assessed the yield in Nanophotometer P300. The process of **Fragmentation and Labeling** ensured that the purified, sense-strand cDNA was fragmented by uracil-DNA glycosylase (UDG) and apurinic/apyrimidinic endonuclease 1 (APE 1) at the unnatural dUTP residues which caused the DNA strand to break. The fragmented cDNA was labeled by terminal deoxynucleotidyl transferase (TdT) using the Affymetrix proprietary DNA Labeling Reagent that is covalently linked to biotin. The fragmentation step required 5.5 µg of single-stranded cDNA which was subsequently fragmented by enzymes in the prepared Fragmentation Master Mix. 45 µl of fragmented ss-cDNA was used in the last step before hybridization – labeling. **Target**

Hybridization Setup for Affymetrix Array Strips required Hybridization Master Mix & Cocktail with fragmented and labeled ss-cDNA to be denatured at 99 °C for 5 min before 120 µl being loaded in the middle of wells of hybridization tray. After that, the array strip was placed into the hybridization tray containing the hybridization cocktail samples and both were placed in the GeneAtlas Hybridization Station for 16-hour incubation at 48 °C.

Microarray experiments were performed using the Rat Gene 2.1 ST Array Strip in quadruplicate (per strain/programming). The whole hybridization procedure was performed using the Affymetrix GeneAtlas® system according to manufacturer's instructions. The quality control of the chips was performed using Affymetrix Expression Console. Partek Genomics Suite (Partek, St. Louis, Missouri) was used for subsequent data analysis. After applying quality filters and data normalization by Robust Multichip Average (RMA) algorithm implemented in Affymetrix Expression Console, the set of obtained differentially expressed probesets was filtered by false discovery rate (FDR) method implemented in PARTEK Genomics Suite 6.6 (Partek, St. Louis, Missouri). Transcriptomic data were then processed by standardized sequence of analyses (hierarchical clustering and principal component analysis, gene ontology, gene set enrichment, "Upstream Regulator Analysis", "Mechanistic Networks", "Causal Network Analysis" and "Downstream Effects Analysis") using Ingenuity Pathway Analysis (Qiagen).

4.4.7.4. Quantitative PCR

4.4.7.4.1. RNA reverse transcription to cDNA

Total RNA (1 µg) was reverse-transcribed with oligo-dT primers using the SuperScript III (Invitrogen).

We used total RNA (c = 250 ng/µl) isolated from liver, white and brown fat to prepare cDNA for the quantitative PCR validation of the microarray data. First step included 1 µl of Oligo $(dT)_{12\text{-}18}$ Primers (Life Technologies), 1 µl of 10 mM dNTP mix (Life Technologies) and 1 µg of RNA (4 µl of c = 250 ng/µl) with nuclease-free water in total volume of 13 µl per reaction. Tubes were incubated for 5 min at 65 °C in PCR thermal cycler Labcycler (SensoQuest). The second step of the reaction included 1 µl of DTT (DL-Dithiothreitol, Clelands agent) for stabilization of the enzyme, 1 µl RNaseOUT^{TM} recombinant ribonuclease

inhibitor to avoid RNA degradation, 4 µl of 5x Buffer and 1 µl of SuperScript™ III reverse transcriptase in total volume of 7 µl. In the process we also prepared 2 negative controls, 1NC where the RNA was present but no enzyme added and 2NC without RNA, but with added enzyme. We combined both reaction mixes in total volume of 20 µl and incubated the samples and negative controls in thermal cycler for 1 hour at 50 °C followed by 15 min at 70 °C for the synthesis of cDNA. We diluted the resulting 20 µl with 180 µl of nuclease-free water and obtained 200 µl of cDNA with c = 5 ng/µl to be used in quantitative PCR validation.

To validate the efficiency of the reverse transcription we performed β-actin (beta actin) PCR using Phusion® High-Fidelity DNA polymerase (Thermo Scientific®). We added third negative control 3NC, where only the reaction mix and 2 µl of nuclease-free water was used instead of sample.

4.4.7.4.2. Real-Time PCR

To validate microarray gene expression data, we performed quantitative real-time PCR (RT-qPCR) using TaqMan® probes (Applied Biosystems™) according to the manufacturer´s instruction. Real-time PCR reaction was performed in triplicate with TaqMan® Gene Expression Master Mix (Applied Biosystems) according to the manufacturer's protocol (Invitrogen) using Applied Biosystems® 7900HT Real-Time PCR System. The probes used for validation were Rn00567167_m1 (*Hsd11b1*), Rn00562884_m1 (*Cox8b*), Rn00566938_m1 (*Sod1*), Rn00581867_m1 (*Dio2*), Rn00593680_m1 (*Apoe*), Rn00589173_m1 (*Nr0b2*), Rn01416753_m1 (*Pcsk9*), Rn01495769_m1 (*Srebf1*), Rn01789864_s1 (*Acot1*), Rn00567532_m1 (*Sqle*), Rn00562597_m1 (*Slc2a4*), Rn01454585_g1 (*Vegfb*), Rn00567668_m1 (*Adora1*) with FAM-MGB reporter dyes (FAM – 6-carboxyfluorescein, MGB – minor groove binder) with 3' nonfluorescent quencher (NFQ) for lower background signal and better quantitation. Results were analyzed using the Livak analysis method [294] with glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) as reference gene.

4.5. Statistical analysis

All statistical analyses were performed using STATISTICA 13.3 (TIBCO Software Inc.). When comparing morphometric and biochemical variables between groups, two-way ANOVA with STRAIN and DIET as major factors were used, followed by post-hoc Fisher's test for comparison of the specific pairs of variables. Null hypothesis was rejected whenever $p>0.05$.

5. Results

5.1. Chapter 1 – Study 1

5.1.1. Mothers (STD/STD, HSD/HSD)

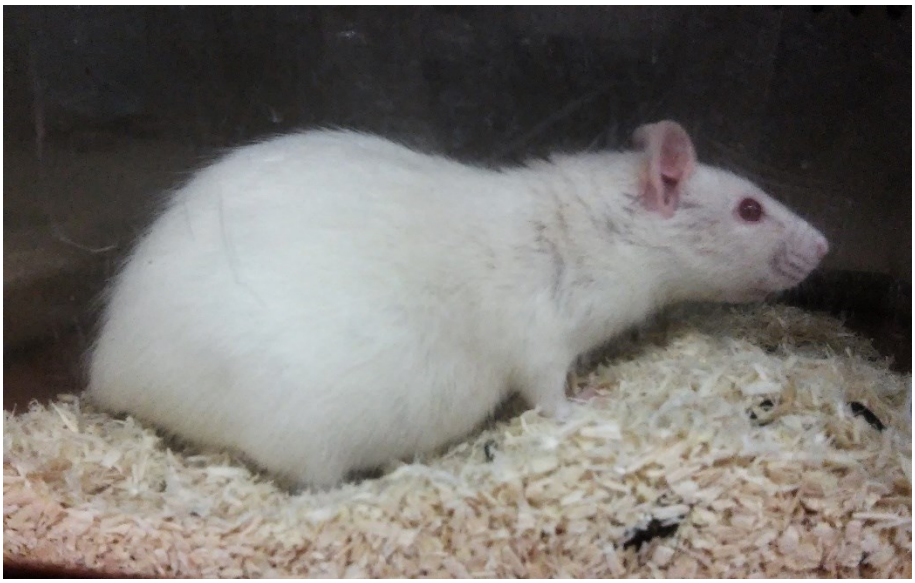


Fig. 4. Pregnant SHR female.

5.1.1.1. Body weight and diet consumption

Body weight of the females was measured for at least 11 weeks throughout the experiment. Control groups of female rats of both strains showed similar weight during the whole period of measurement, as opposed to HSD-fed females showing a significant decrease in body weight after delivery.

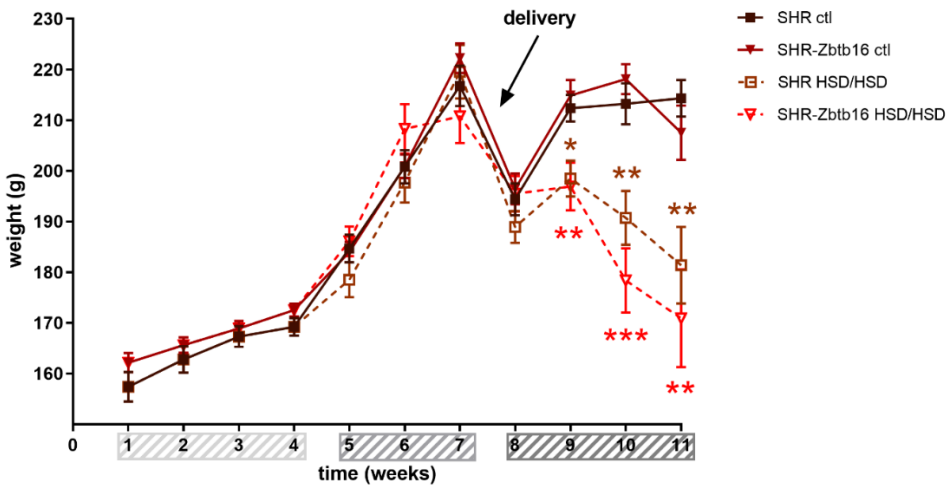


Fig. 5. Body weight measurements of F0 SHR and SHR-*Zbtb16* adult female rats from 16 weeks of age (weeks 1-4), in gravidity (weeks 5-7) and lactation period (weeks 8-11), SHR control females (dark brown squares, full line), SHR females fed HSD during pregnancy (light brown empty squares, dashed line), SHR-*Zbtb16* control females (dark red triangles, full line), SHR-*Zbtb16* females fed HSD during pregnancy (red empty triangles, dashed line). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and DIET as major factors) Fisher's test of pairwise group comparison within each strain are indicated as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Significant differences between SHR females fed STD and SHR females fed HSD are represented by light brown asterisks (*) – week 9 *, week 10 **, week 11 **. Significant differences between SHR-Zbtb16 females fed STD and SHR-Zbtb16 females fed HSD are represented by red asterisks (*) – week 9 **, week 10 ***, week 11 **.

Although the diets provided similar amount of calories and HSD-fed females consumed larger amount of the diet during pregnancy (Fig. 6), their body weight did not differ significantly from STD-fed females (Fig. 5). We observed no significant differences in diet intake during breastfeeding period, although body weight of both experimental groups declined.

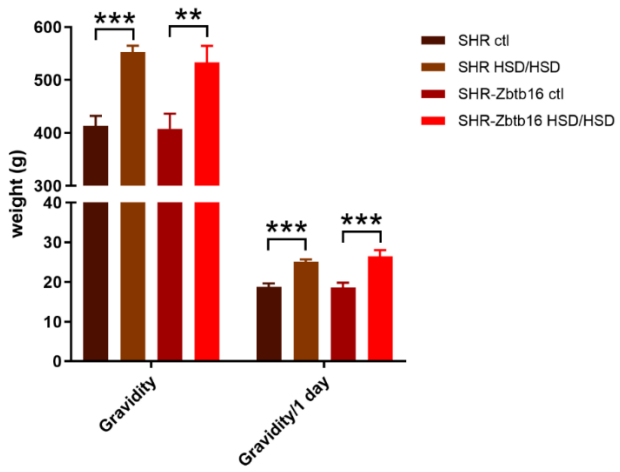
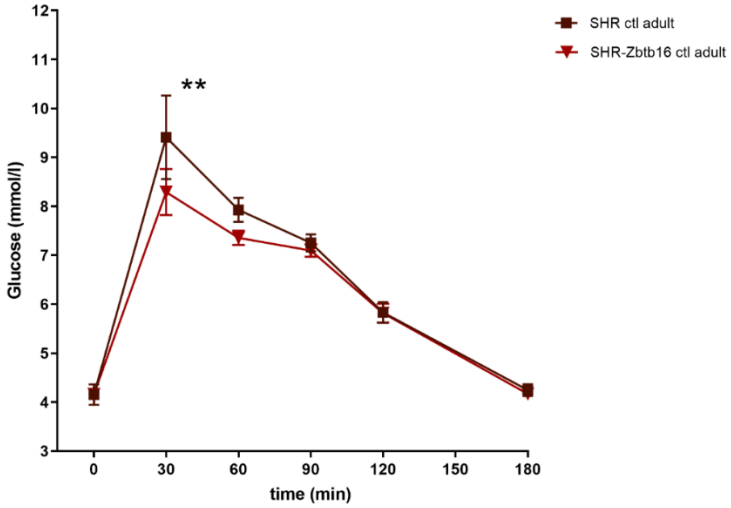


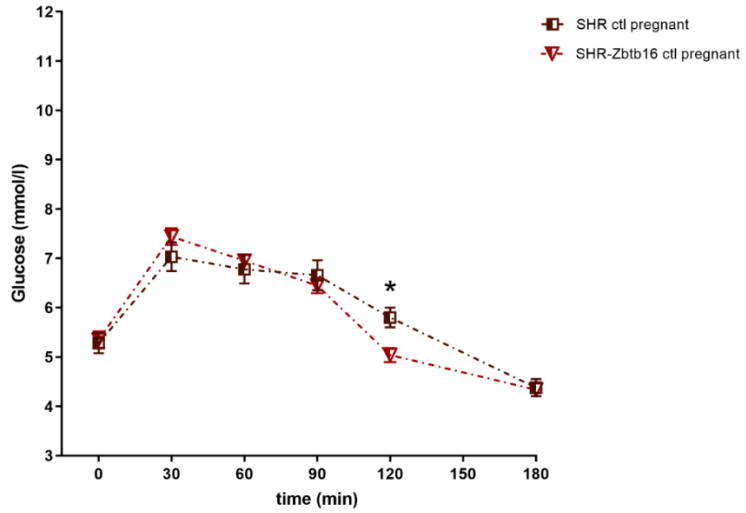
Fig 6. Diet consumption in gravidity of F0 SHR and SHR-*Zbtb16* female rats, SHR control females (dark brown bars), SHR females fed HSD during pregnancy (light brown bars), SHR-*Zbtb16* control females (dark red bars), SHR-*Zbtb16* females fed HSD during pregnancy (red bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and DIET as major factors) Fisher's test of pairwise group comparison within each strain are indicated as follows: ** $p<0.01$, *** $p<0.001$.

5.1.1.2. Metabolic profile

SHR rats of control group showed decreased glucose tolerance compared to SHR-Zbtb16 female rats prior to pregnancy (Fig. 7).



The difference in glucose tolerance between the strains minimized during pregnancy of control groups.



The strain differences became apparent again mid-pregnancy of females fed HSD, when SHR females showed decreased glucose tolerance as a response to HSD-feeding.

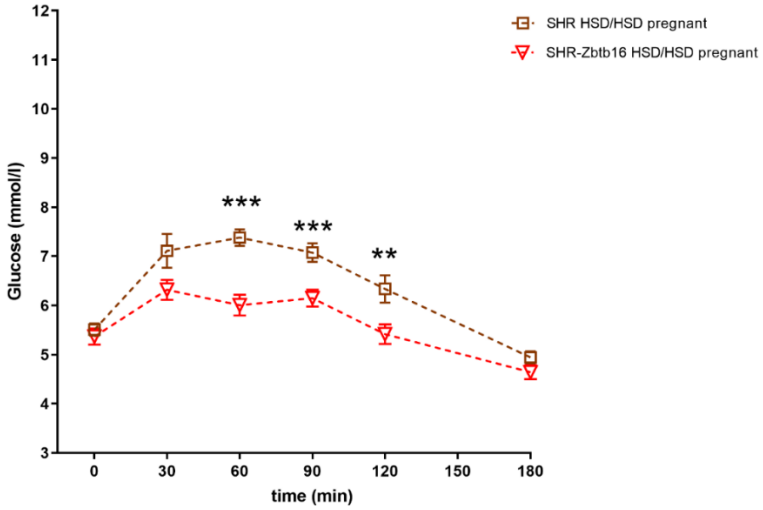


Fig. 7. The oral glucose tolerance test (OGTT). The course of glycaemic curves in F0 rat dams in adulthood before and during pregnancy. SHR control females in adulthood (dark brown squares, full line), SHR females fed STD during pregnancy (dark brown half empty squares, dashed line), SHR females fed HSD during pregnancy (light brown empty squares, dashed line), SHR-*Zbtb16* control females in adulthood (dark red triangles, full line), SHR-Zbtb16 females fed STD during pregnancy (dark red half empty squares, dashed line), SHR-*Zbtb16* females fed HSD during pregnancy (red empty triangles, dashed line). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and DIET as major factors) Fisher's test of pairwise group comparison within each strain are indicated as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

In order to compare the courses of glycaemic curves in each strain I've included graphs showing significant differences in glucose tolerance of control non-pregnant, control pregnant and HSD-fed pregnant females. In SHR females, the pregnant control group had the lowest levels of blood glucose (Fig. 8) as opposed to SHR-Zbtb16 females, in which the HSD-fed pregnant females showed the lowest levels of blood glucose (Fig. 9).

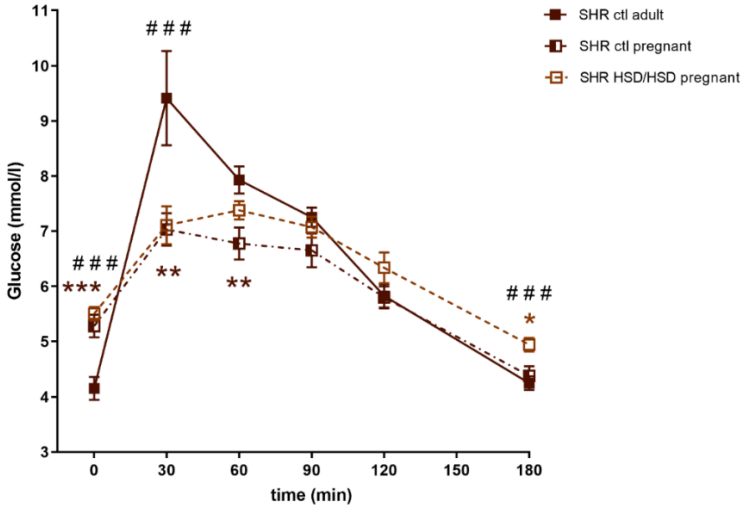


Fig. 8. The oral glucose tolerance test (OGTT). The course of glycaemic curves in F0 rat dams in adulthood before and during pregnancy. SHR control females in adulthood (dark brown squares, full line), SHR females fed STD during pregnancy (dark brown half empty squares, dashed line), SHR females fed HSD during pregnancy (light brown empty squares, dashed line). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for DIET as major factor) Fisher's test comparison of HSD-fed vs. control females are indicated as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Effect of pregnancy in SHR females is represented by dark brown asterisks (*) – significant differences between non-pregnant SHR females fed STD and pregnant SHR females fed STD, t=0 ***, t=30 **, t=60 **; effect of diet is represented by light brown asterisks (*) – significant differences between pregnant SHR females fed STD and pregnant SHR females fed HSD, t=180 *. # represents differences between non-pregnant SHR females fed STD and pregnant SHR females fed HSD, t=0 min ***, t=30 min ***, t=180 min ***.

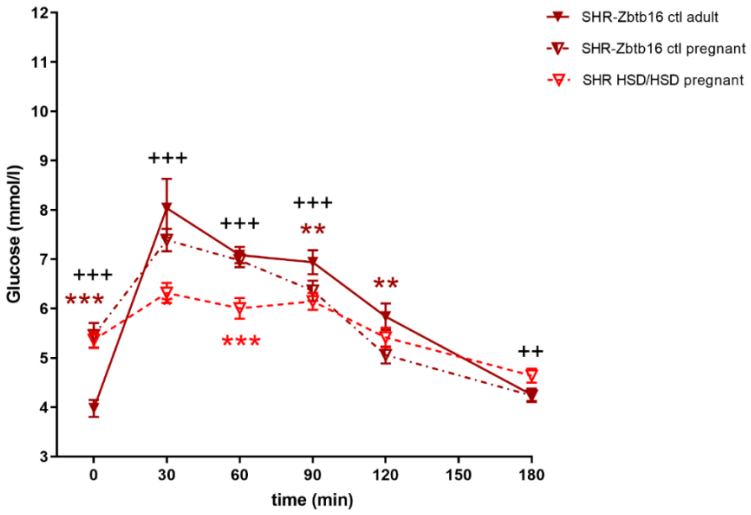


Fig. 9. The oral glucose tolerance test (OGTT). The course of glycaemic curves in F0 rat dams in adulthood before and during pregnancy. SHR-Zbtb16 control females in adulthood (dark red triangles, full line), SHR-Zbtb16 females fed STD during pregnancy (dark red half empty triangles, dashed line), SHR-Zbtb16 females fed HSD during pregnancy (red empty triangles, dashed line). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for DIET as major factor) Fisher's test comparison of HSD-fed vs. control females are indicated as follows: ** $p<0.01$, *** $p<0.001$. Effect of pregnancy in SHR-Zbtb16 females is represented by dark red asterisks (*) – significant differences between non-pregnant SHR-Zbtb16 females fed STD and pregnant SHR-Zbtb16 females fed STD, t=0 ***, t=90 **, t=120 **; effect of diet is represented by red asterisks (*) – significant differences between pregnant SHR-Zbtb16 females fed STD and pregnant SHR-Zbtb16 females fed HSD, t=60 ***. + represents differences between non-pregnant SHR-Zbtb16 females fed STD and pregnant SHR-Zbtb16 females fed HSD, t=0 min ***, t=30 min ***, t=60 min ***, t=90 ***, t=180 min **.

The effect of pregnancy and HSD feeding resulted in both strains also in smaller areas under the glycaemic curves, respectively (Fig. 10).

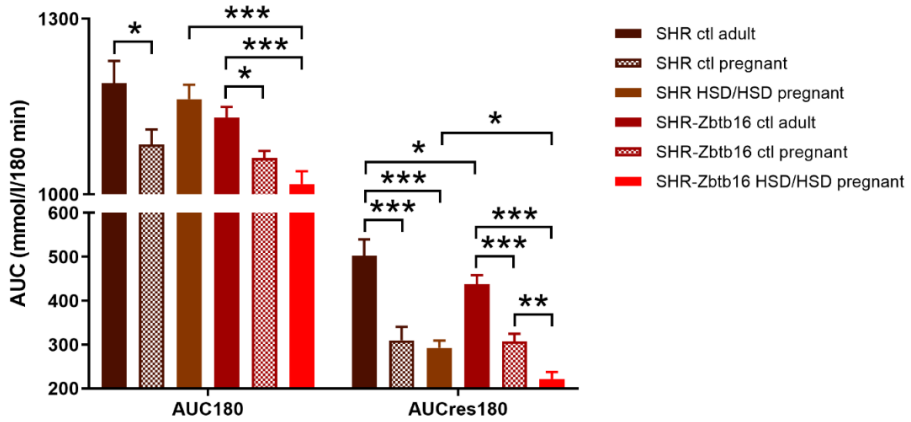


Fig. 10. Area under the curve. Area under the glycaemic curve values calculated from the course of glycaemic curves from 0 to 180 (AUC180) and value of the residual area under the curve (AUCres180). SHR non-pregnant control females (dark brown bars), SHR females fed STD during pregnancy (dark brown patterned bars), SHR females fed HSD during pregnancy (light brown bars), SHR-*Zbtb16* non-pregnant control females (dark red bars), SHR-*Zbtb16* fed STD during pregnancy (dark red patterned bars), SHR-*Zbtb16* females fed HSD during pregnancy (red bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and DIET as major factors) Fisher's test of pairwise group comparison within each strain are indicated as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

In SHR females we observed a significant increase in fasting serum insulin levels caused by HSD-feeding in pregnancy, as opposed to SHR-Zbtb16 females, whose serum insulin levels have not differed from not pregnant control group (Fig. 11).

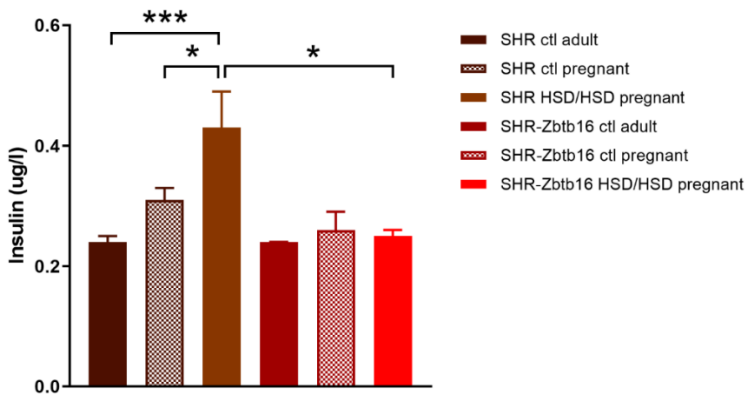


Fig. 11. Fasting insulin concentrations in SHR non-pregnant control females (dark brown bars), SHR females fed STD during pregnancy (dark brown patterned bars), SHR females fed HSD during pregnancy (light brown bars), SHR-*Zbtb16* non-pregnant control females (dark red bars), SHR-*Zbtb16* fed STD during pregnancy (dark red patterned bars), SHR-*Zbtb16* females fed HSD during pregnancy (red bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and DIET as major factors) Fisher's test of pairwise group comparison within each strain are indicated as follows: * $p<0.05$, *** $p<0.001$.

Prior to pregnancy, the concentrations of cytokines were comparable between the two strains except for the levels of interleukin 6, interferon gamma (Fig. 12) and pancreatic polypeptide (Fig. 13) that were slightly higher in SHR-Zbtb16 females. In both strains, the pregnancy increased levels of leptin (Fig. 13) and in SHR-Zbtb16 decreased the concentrations of interleukin 17 (Fig. 12). Pregnant control SHR females had increased interleukin 18 (Fig.

12) and decreased polypeptide YY (Fig. 13) compared to pregnant control SHR-Zbtb16 females. Exposure to HSD in pregnancy led to an elevation of vascular endothelial growth factor (Fig. 12) and pancreatic polypeptide (Fig. 13) only in SHR experimental group.

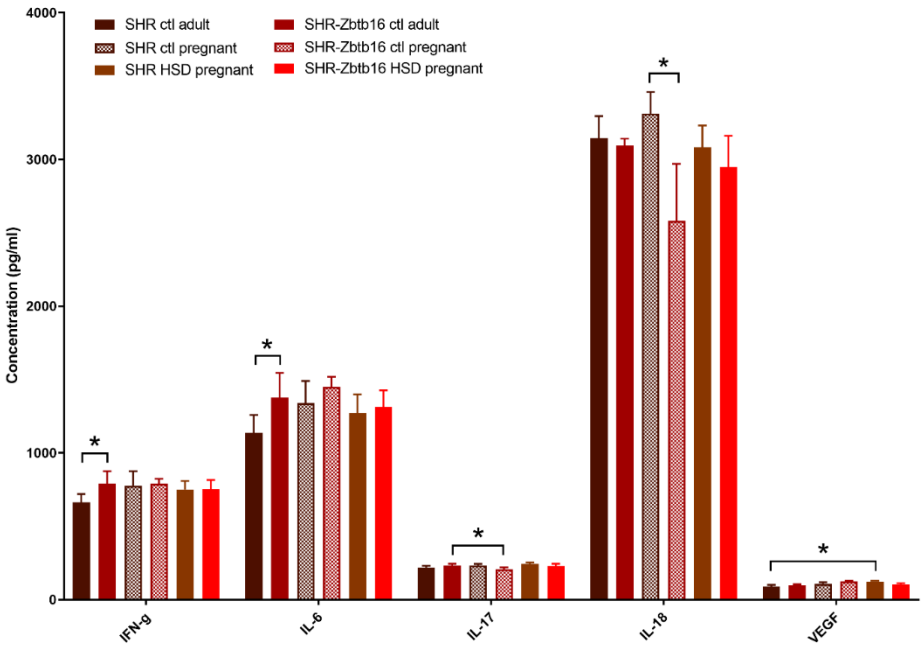


Fig. 12. Cytokine profile. Cytokine concentrations in the serum of SHR non-pregnant control females (dark brown bars), SHR females fed STD during pregnancy (dark brown patterned bars), SHR females fed HSD during pregnancy (light brown bars), SHR-*Zbtb16* non-pregnant control females (dark red bars), SHR-*Zbtb16* fed STD during pregnancy (dark red patterned bars), SHR-*Zbtb16* females fed HSD during pregnancy (red bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and DIET as major factors) Fisher's test of pairwise group comparison within each strain are indicated as follows: * $p<0.05$.

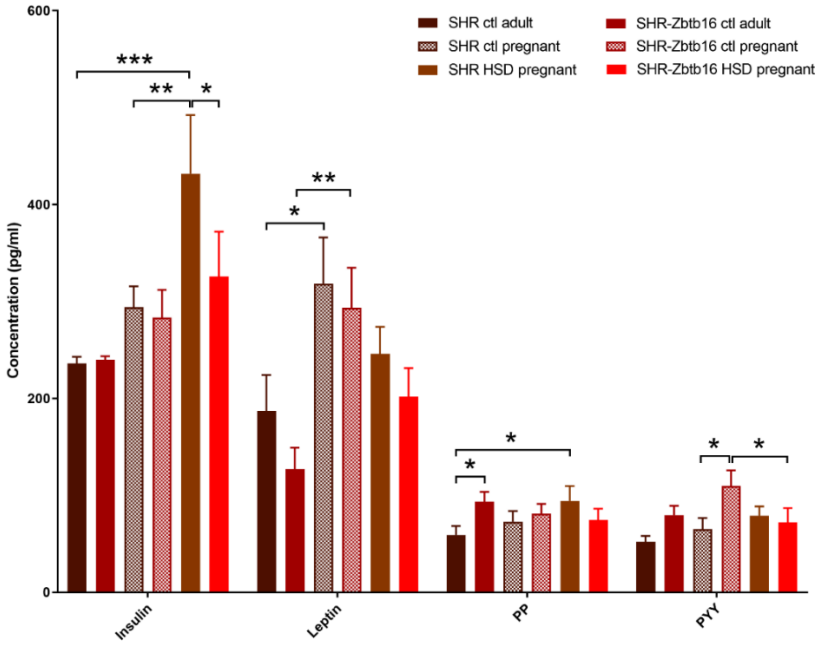
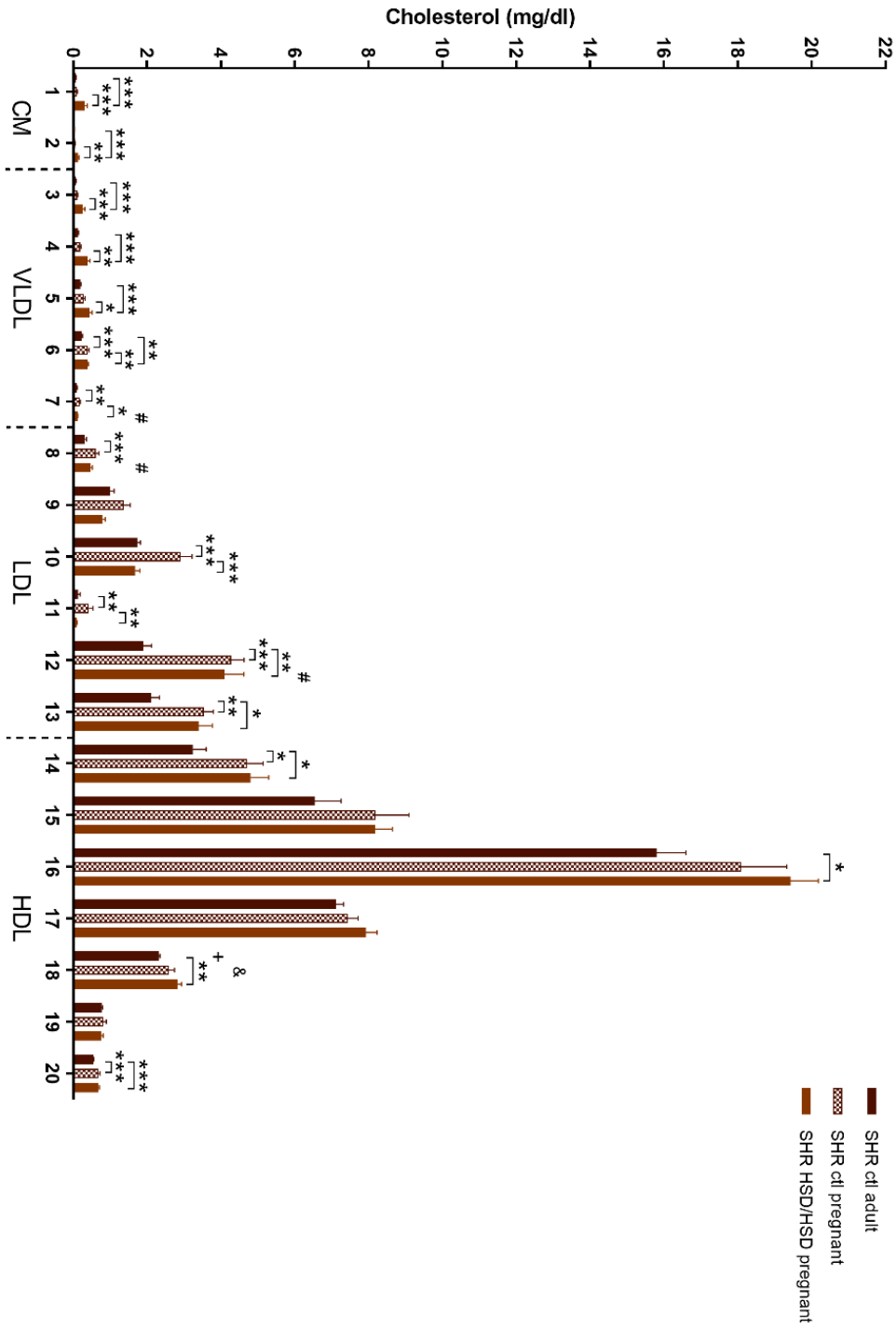


Fig. 13. Hormone profile. Hormone concentrations in the serum of SHR non-pregnant control females (dark brown bars), SHR females fed STD during pregnancy (dark brown patterned bars), SHR females fed HSD during pregnancy (light brown bars), SHR-*Zbtb16* non-pregnant control females (dark red bars), SHR-*Zbtb16* fed STD during pregnancy (dark red patterned bars), SHR-*Zbtb16* females fed HSD during pregnancy (red bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and DIET as major factors) Fisher's test of pairwise group comparison within each strain are indicated as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

5.1.1.3. Lipid profile

The distribution of cholesterol into individual lipoprotein classes showed little significant differences between the control groups, both not pregnant and pregnant in small HDL cholesterol levels and differed only slightly between pregnant experimental groups – an increase of very small LDL cholesterol level in SHR-Zbtb16. The pregnancy itself significantly increased cholesterol levels in most lipoprotein classes of both pregnant control groups. HSD in pregnancy increased cholesterol content in chylomicron and very low density lipoprotein fractions (Fig. 14).


Cholesterol (mg/dl)
0
2
4
6
8
10
12
14
16
18
20
22
CM
VLDL
LDL
HDL
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
SHR ctl adult
SHR ctl pregnant
SHR HSD/HSD pregnant

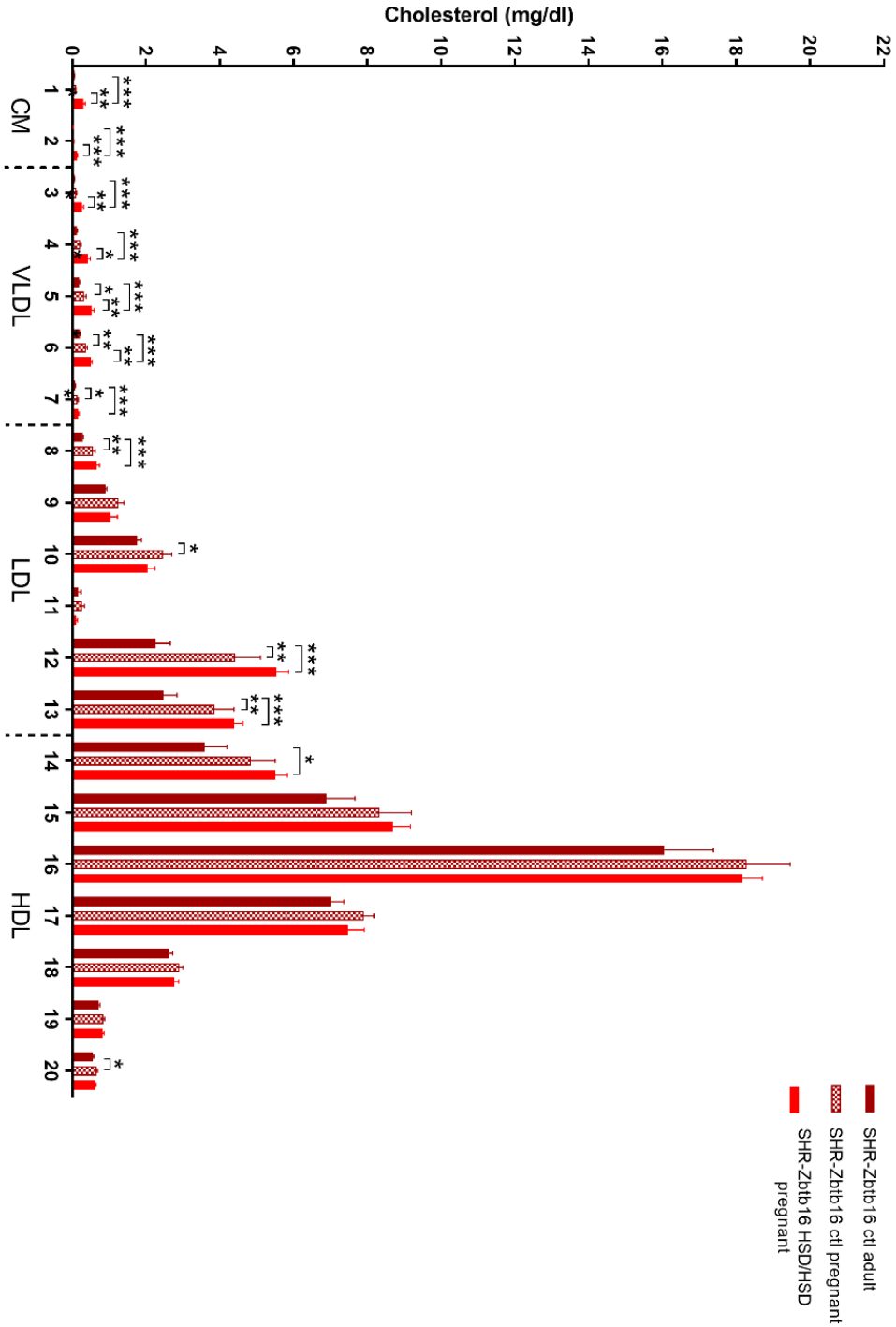
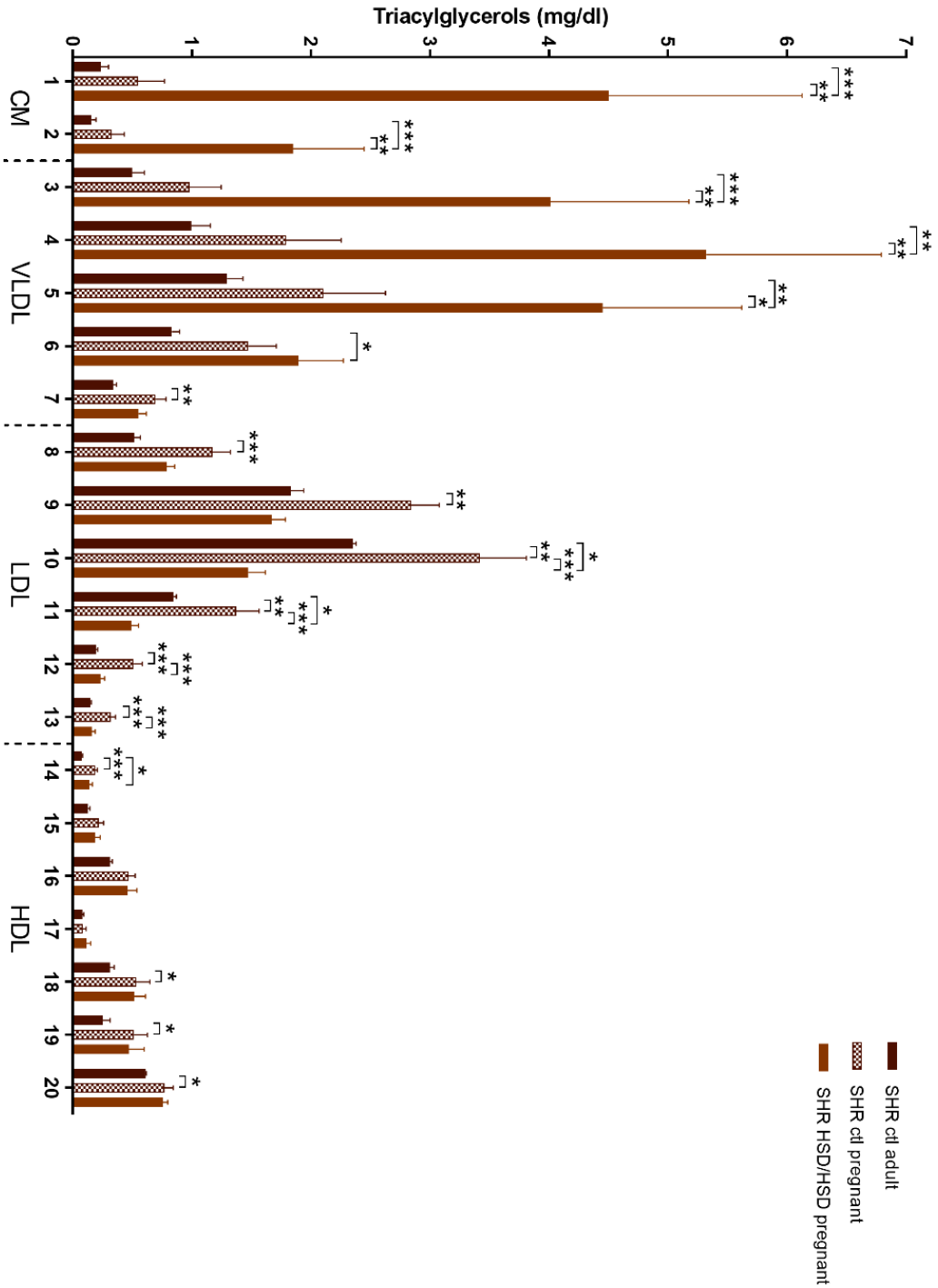


Fig. 14. Cholesterol profile. The cholesterol (C) content in 20 lipoprotein subfractions in SHR and SHR-Zbtb16 female rats. SHR non-pregnant control females (dark brown bars), SHR females fed STD during pregnancy (dark brown patterned bars), SHR females fed HSD during pregnancy (light brown bars), SHR-*Zbtb16* non-pregnant control females (dark red bars), SHR-*Zbtb16* fed STD during pregnancy (dark red patterned bars), SHR-*Zbtb16* females fed HSD during pregnancy (red bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and DIET as major factors) Fisher's test of pairwise group comparison within each strain are indicated as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. + represents strain differences between non pregnant control SHR and non pregnant control SHR-Zbtb16 females, C18 *. &

represents strain differences between pregnant control SHR and pregnant control SHR-Zbtb16 females, C18 *. # represents strain differences between HSD-fed pregnant SHR females and HSD-fed pregnant SHR-*Zbtb16*, C7 *, C8 *, C12 *. The allocation of individual lipoprotein subfractions to major lipoprotein classes is shown in order of particle's decreasing size from left to right. CM-chylomicron, VLDL-very low density lipoprotein, LDL-low density lipoprotein, HDL-high density lipoprotein.

The distribution of triacylglycerol into individual lipoprotein classes showed no significant differences between the strains. The pregnancy itself significantly increased triacylglycerol levels in most lipoprotein classes of both pregnant control groups. HSD in pregnancy significantly increased triacylglycerol content in chylomicron fraction, similar to cholesterol distribution. Interestingly, LDL triacylglycerol levels were significantly increased only in pregnant control females fed STD (Fig. 15).


Triacylglycerols (mg/dl)
0
1
2
3
4
5
6
7
CM
VLDL
LDL
HDL
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
SHR ctl adult
SHR ctl pregnant
SHR HSD/HSD pregnant

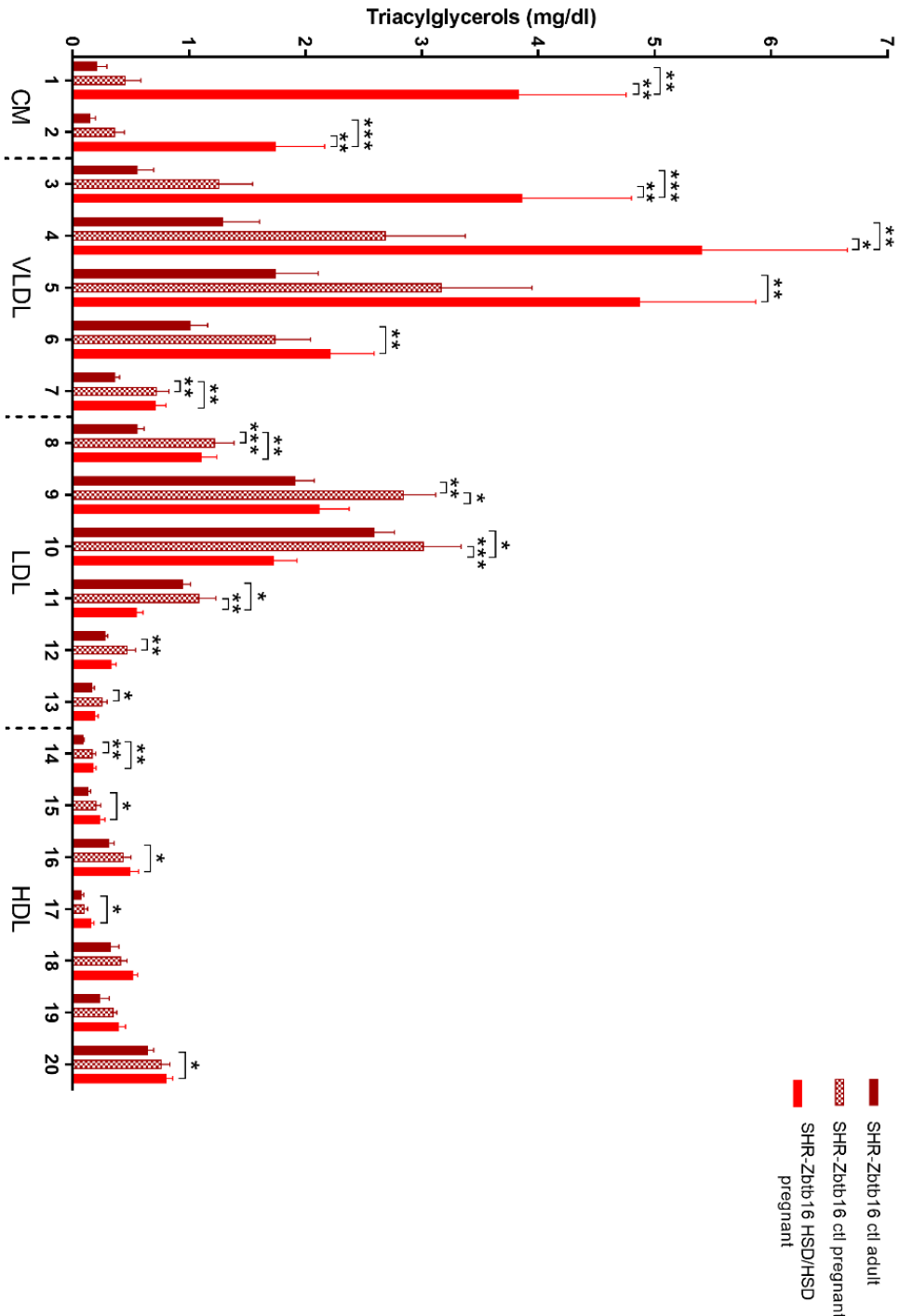


Fig. 15. Triacylglycerol profile. The triacylglycerol (TG) content in 20 lipoprotein subfractions in SHR and SHR-Zbtb16 female rats. SHR non-pregnant control females (dark brown bars), SHR females fed STD during pregnancy (dark brown patterned bars), SHR females fed HSD during pregnancy (light brown bars), SHR-*Zbtb16* non-pregnant control females (dark red bars), SHR-*Zbtb16* fed STD during pregnancy (dark red patterned bars), SHR-*Zbtb16* females fed HSD during pregnancy (red bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and DIET as major factors) Fisher's test of pairwise group comparison within each strain are indicated as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. The allocation of individual lipoprotein subfractions to major lipoprotein classes is shown in order of particle's decreasing size from

left to right. CM-chylomicron, VLDL-very low density lipoprotein, LDL-low density lipoprotein, HDL-high density lipoprotein.

5.1.2. F1 offspring

Fig. 16. F1 generation pups (approx. 2 weeks of age).

5.1.2.1. Morphometry

Morphometric analysis of 6 months old male offspring showed comparable body weights, slightly higher in SHR controls. Thereupon we observed higher absolute (Fig. 17) and relative (Fig. 18) weights of liver, retroperitoneal and epididymal fat pads, interscapular brown fat (Fig. 20, 21) and hind leg *musculus soleus* (Fig. 22) in SHR control males. Due to polydactyly and hind leg aberrations in SHR-Zbtb16, the differences in this particular muscle mass will only be described compared to control in each strain separately, as the difference with SHR is likely due to *Lx* polymorphism.

Metabolic programming with HSD decreased relative weight of kidneys (Fig. 18) in both strains and increased relative weights of interscapular brown fat (Fig. 21) by 46.5% in

SHR (0.065545±0.004 vs 0.0959968±0.005 g/100 g BW) and by 70% in SHR-Zbtb16 (0.053475±0.004 vs 0.090943±0.004 g/100 g BW). In SHR-Zbtb16 exclusively the metabolic programming HSD/HSD increased relative muscle mass in m. soleus (Fig. 22).

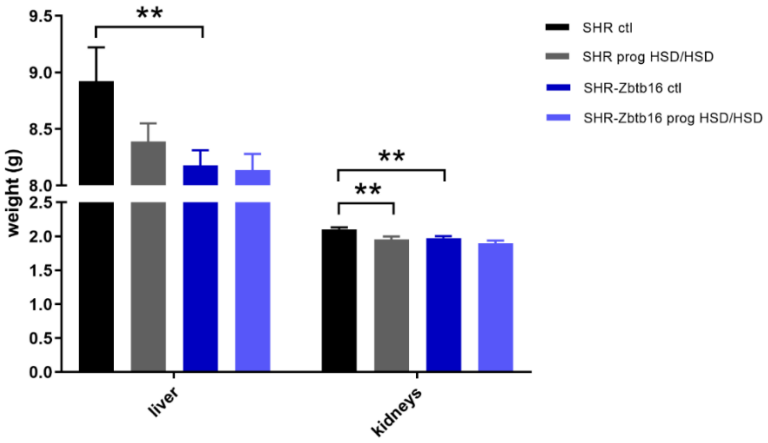


Fig 17. Liver and kidney weight in adult F1 SHR control males (black bars), SHR males programmed with maternal HSD both in pregnancy and lactation (grey bars), SHR-Zbtb16 control males (dark blue bars) and SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation (blue bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and MATERNAL DIET as major factors) Fisher's test of pairwise group comparison (control vs programmed males) within each strain are indicated as follows: ** $p<0.01$.

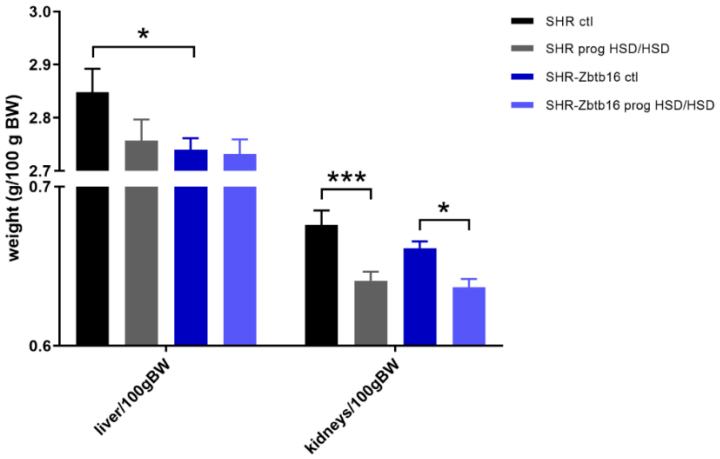


Fig. 18. Liver and kidney weight per 100g of body weight in adult F1 SHR control males (black bars), SHR males programmed with maternal HSD both in pregnancy and lactation (grey bars), SHR-Zbtb16 control males (dark blue bars) and SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation (blue bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN

and MATERNAL DIET as major factors) Fisher's test of pairwise group comparison (control vs programmed males) within each strain are indicated as follows: * $p<0.05$, *** $p<0.001$.

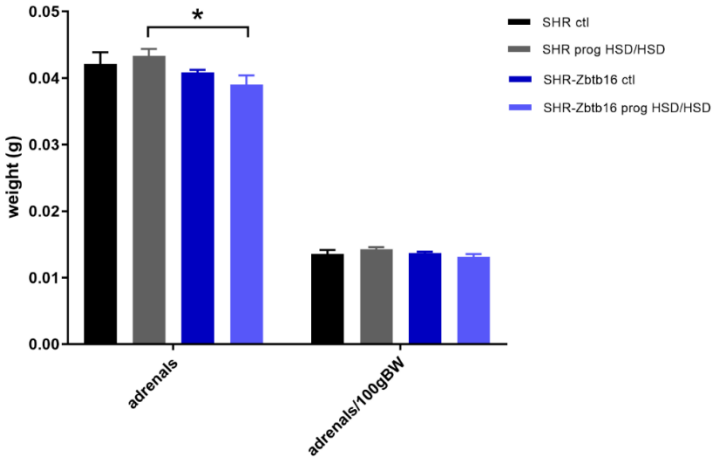


Fig. 19. Adrenal weight in adult F1 SHR control males (black bars), SHR males programmed with maternal HSD both in pregnancy and lactation (grey bars), SHR-Zbtb16 control males (dark blue bars) and SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation (blue bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and MATERNAL DIET as major factors) Fisher's test of pairwise group comparison (control vs programmed males) within each strain are indicated as follows: * $p<0.05$.

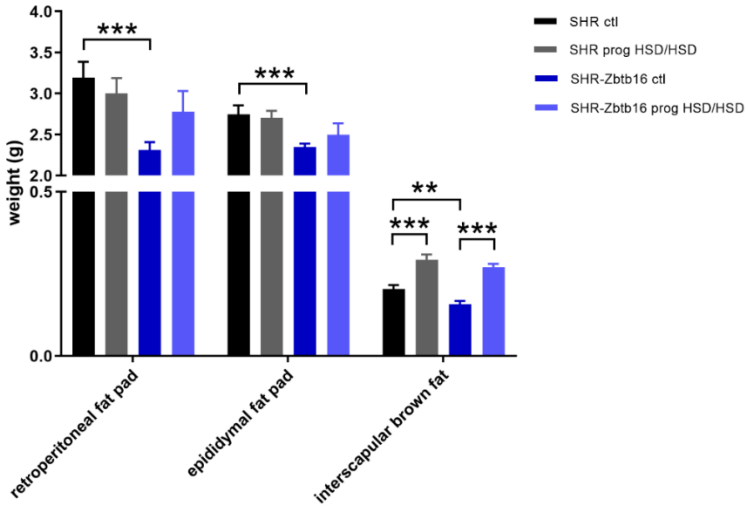


Fig. 20. Retroperitoneal fat pad, epididymal fat pad and interscapular brown fat weight in adult F1 SHR control males (black bars), SHR males programmed with maternal HSD both in pregnancy and lactation (grey bars), SHR-

Zbtb16 control males (dark blue bars) and SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation (blue bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and MATERNAL DIET as major factors) Fisher's test of pairwise group comparison (control vs programmed males) within each strain are indicated as follows: ** p<0.01, *** p<0.001.

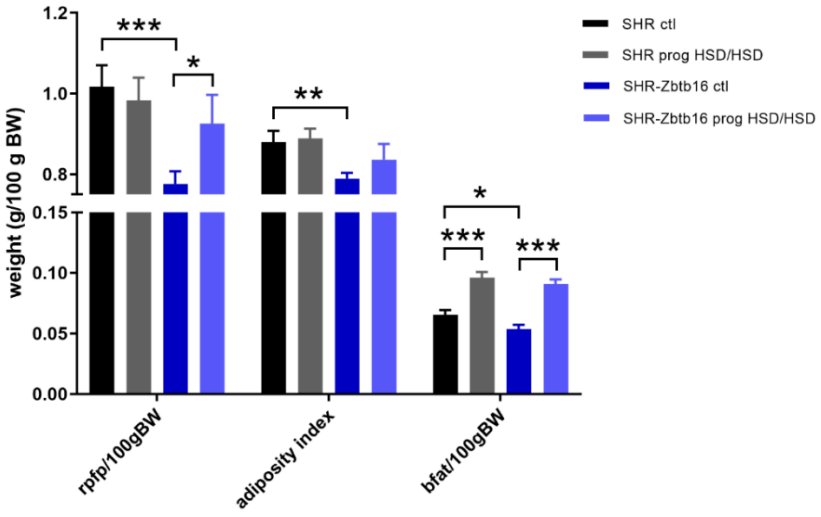


Fig. 21. Retroperitoneal fat pad, epididymal fat pad (adiposity index) and interscapular brown fat weight per 100 g of body weight in adult F1 SHR control males (black bars), SHR males programmed with maternal HSD both in pregnancy and lactation (grey bars), SHR-Zbtb16 control males (dark blue bars) and SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation (blue bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and MATERNAL DIET as major factors) Fisher's test of pairwise group comparison (control vs programmed males) within each strain are indicated as follows: ** p<0.01, *** p<0.001.

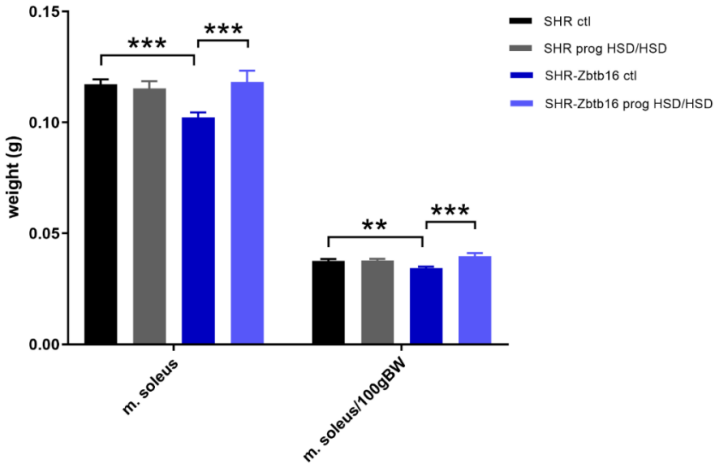


Fig. 22. Hind leg skeletal muscle (musculus soleus) weight in adult F1 SHR control males (black bars), SHR males programmed with maternal HSD both in pregnancy and lactation (grey bars), SHR-Zbtb16 control males (dark blue bars) and SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation (blue bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and MATERNAL DIET as major factors) Fisher's test of pairwise group comparison (control vs programmed males) within each strain are indicated as follows: ** p<0.01, *** p<0.001.

5.1.2.2. Glucose tolerance and insulin levels

During oral glucose tolerance test, SHR-Zbtb16 males showed better glucose tolerance in comparison with respective SHR groups. Programmed SHR males had significantly lower fasting glycaemia and at the same time significantly higher glycaemia 2 hours after the glucose load compared to control SHR and programmed SHR-Zbtb16 males (Fig. 23). Similarly, the area under the curve (Fig. 24) has increased dramatically in SHR programmed males, but not in SHR-Zbtb16 males which showed no difference in glucose tolerance after HSD/HSD programming. We identified STRAIN*MATERNAL DIET interactions for fasting glycaemia (p=0.0013), t=120 min (p=0.0405) and AUCres180 (p=0.0009).

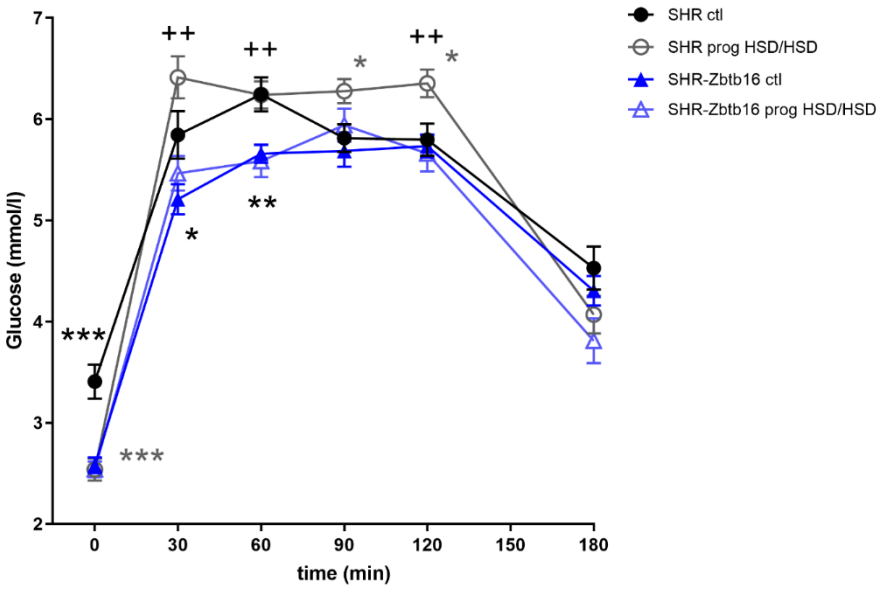


Fig. 23. The oral glucose tolerance test (OGTT). The course of glycaemic curves in F1 adult SHR control males (black circles, full line), SHR males programmed with maternal HSD both in pregnancy and lactation (grey empty circles, full line), SHR-Zbtb16 control males (dark blue triangles) and SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation (blue empty triangles, full line). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and MATERNAL DIET as major

factors) Fisher's test of pairwise group comparison (control vs programmed males) within each strain are indicated as follows: * p<0.05, ** p<0.01, *** p<0.001. Strain differences between SHR control males and SHR-Zbtb16 control males are represented by black asterisks (*), t=0 min ***, t=30 min *, t=60 min **; SHR HSD/HSD programmed males and SHR-Zbtb16 programmed males are represented by black plus signs (+), t=30 min **, t=60 min **, t=120 min **. Effect of programming in SHR males is represented by grey asterisks (*) – significant differences between control and programmed males, t=0 min ***, t=90 min *, t=120 min *.

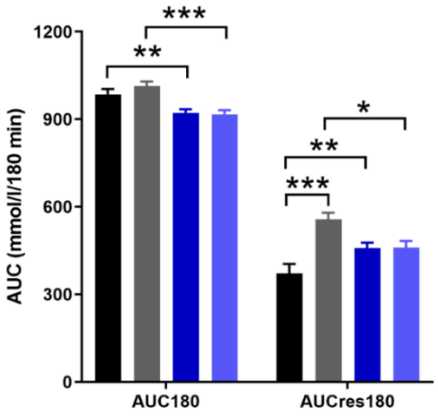


Fig. 24. Area under the curve. Area under the glycaemic curve values calculated from the course of glycaemic curves from 0 to 180 (AUC180) and value of the residual area under the curve (AUCres180). SHR control males (black bars), SHR males programmed with maternal HSD both in pregnancy and lactation (grey bars), SHR-Zbtb16 control males (dark blue bars) and SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation (blue bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and MATERNAL DIET as major factors) Fisher's test of pairwise group comparison (control vs programmed males) within each strain are indicated as follows: * p<0.05, ** p<0.01, *** p<0.001.

Control SHR offspring showed significantly higher fasting insulin concentration compared to control SHR-Zbtb16 rats (Fig. 25). The insulin levels show STRAIN*MATERNAL DIET interactions (p=0.044) as they decreased in SHR but increased in SHR-Zbtb16 as a result of metabolic programming.

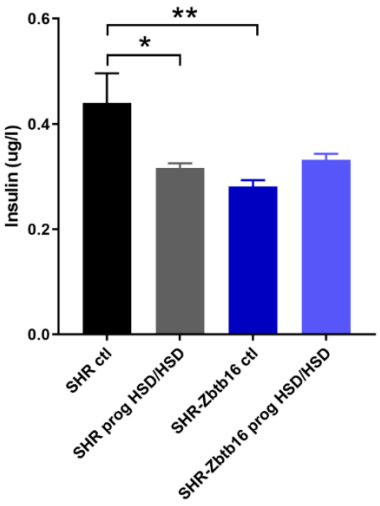


Fig. 25. Fasting insulin concentrations in SHR control males (black bars), SHR males programmed with maternal HSD both in pregnancy and lactation (grey bars), SHR-Zbtb16 control males (dark blue bars) and SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation (blue bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and MATERNAL DIET as major factors) Fisher's test of pairwise group comparison (control vs programmed males) within each strain are indicated as follows: * $p<0.05$, ** $p<0.01$.

5.1.2.3. Lipid profile

Offspring of both strains showed similar profiles of cholesterol and triacylglycerols distribution into lipoprotein fractions. The programming effect of maternal HSD on cholesterol levels was limited to a fraction of very small LDL and medium HDL lipoproteins (Fig. 26).

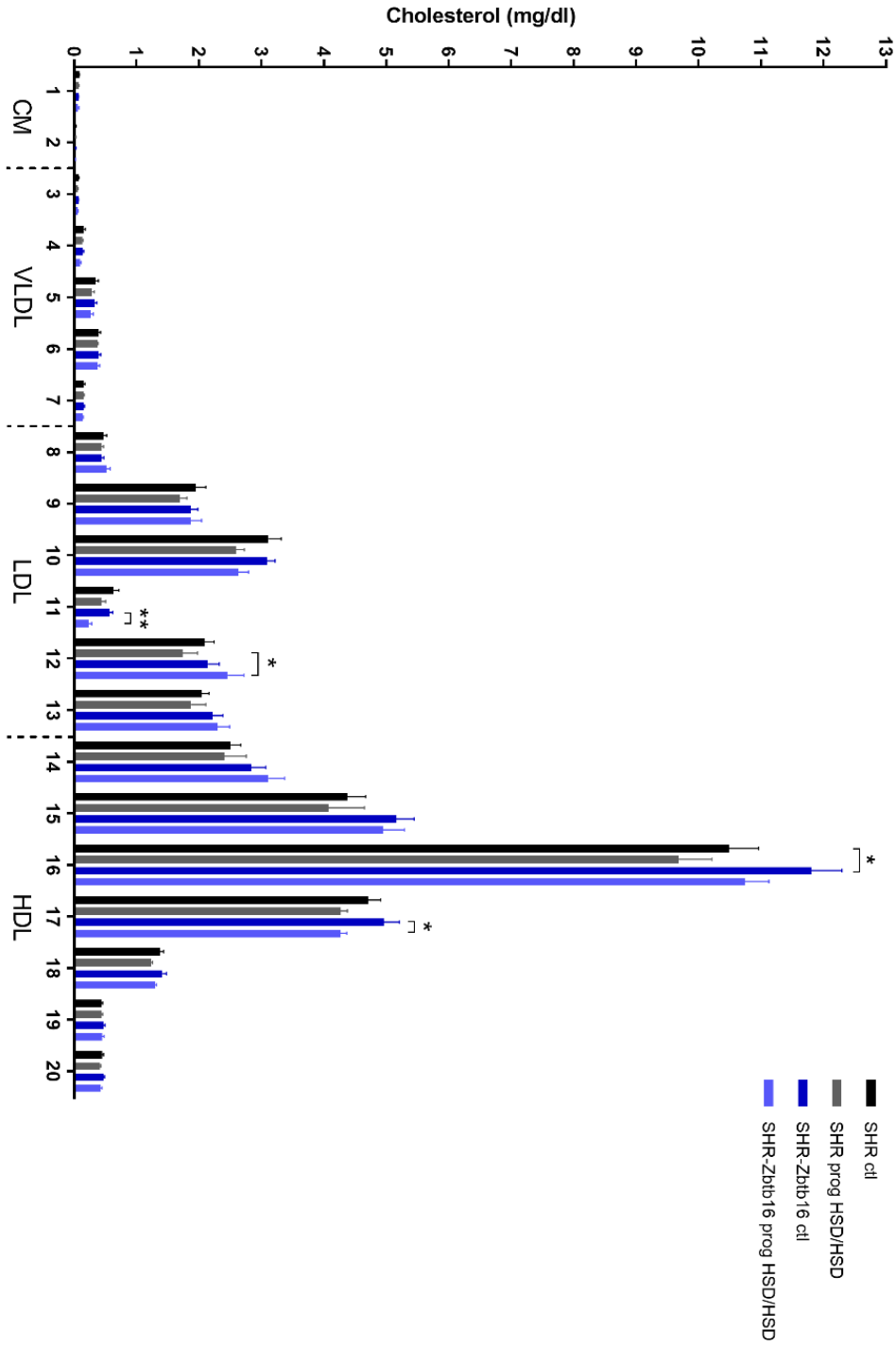


Fig. 26. Cholesterol profile. The cholesterol (C) content in 20 lipoprotein subfractions in SHR control males (black bars), SHR males programmed with maternal HSD both in pregnancy and lactation (grey bars), SHR-Zbtb16 control males (dark blue bars) and SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation (blue bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and MATERNAL DIET as major factors) Fisher's test of pairwise group comparison (control vs programmed males) within each strain are indicated as follows: * $p<0.05$, ** $p<0.01$. The allocation of individual lipoprotein subfractions to major lipoprotein classes is shown in order of particle's decreasing size from

left to right. CM-chylomicron, VLDL-very low density lipoprotein, LDL-low density lipoprotein, HDL-high density lipoprotein.

In HSD-programmed offspring of both strains we observed a reduction in triacylglycerol content of medium, small and very small LDL particles compared to their control groups (Fig. 27). Novel congenic SHR-Zbtb16 programmed by maternal HSD showed a decrease in triacylglycerols in medium HDL particles compared to offspring of STD-fed mothers.

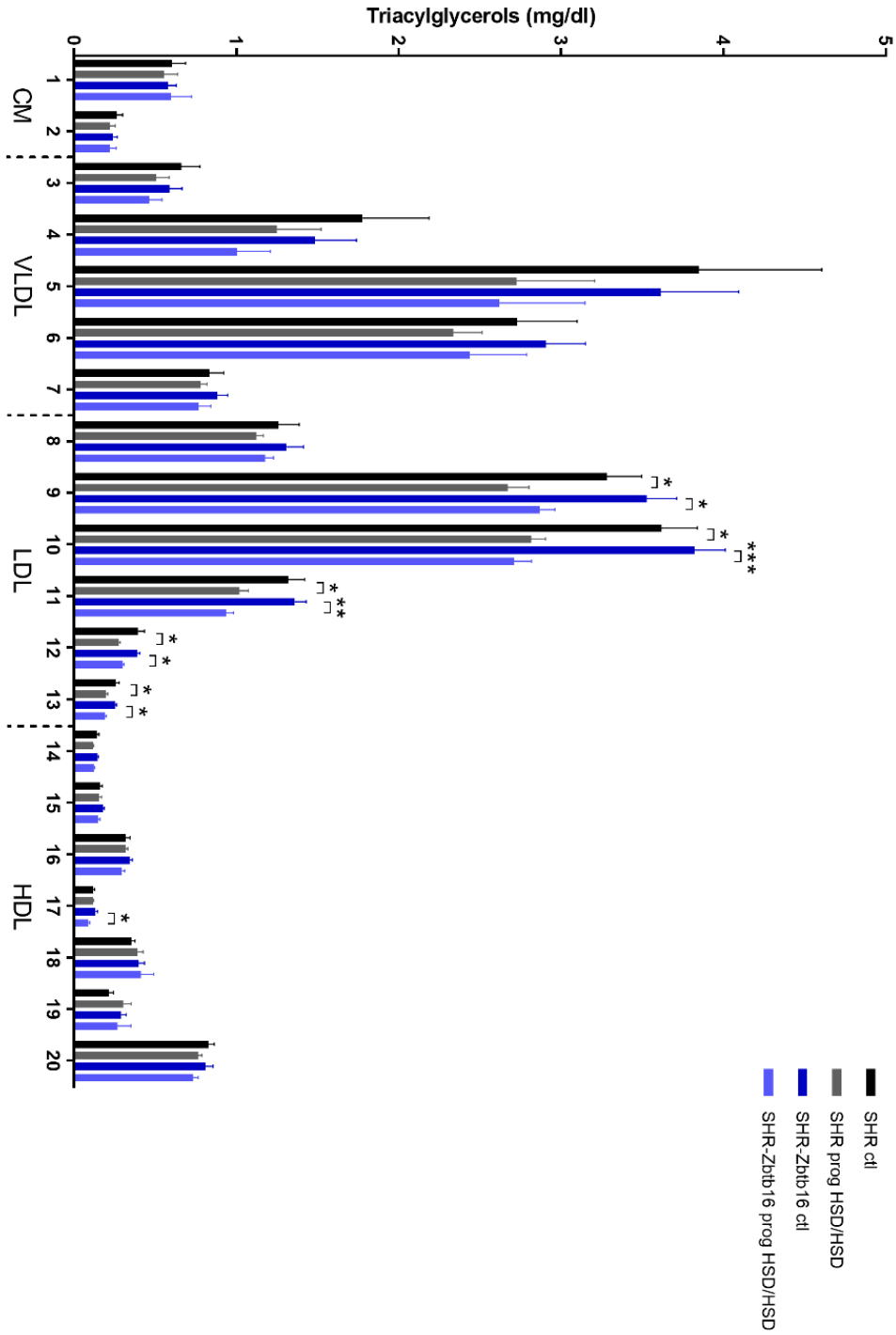


Fig. 27. Triacylglycerol profile. The triacylglycerol (TG) content in 20 lipoprotein subfractions in SHR control males (black bars), SHR males programmed with maternal HSD both in pregnancy and lactation (grey bars), SHR-Zbtb16 control males (dark blue bars) and SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation (blue bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and MATERNAL DIET as major factors) Fisher's test of pairwise group comparison (control vs programmed males) within each strain are indicated as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. The allocation of individual lipoprotein subfractions to major lipoprotein classes is shown in order of particle's

decreasing size from left to right. CM-chylomicron, VLDL-very low density lipoprotein, LDL-low density lipoprotein, HDL-high density lipoprotein.

5.1.2.4. Transcriptomic profile

Transcriptome analysis in liver, brown and white adipose tissues revealed no common transcript among the tissues but limited number of differentially expressed transcripts in SHR control males versus SHR-Zbtb16 control males. HSD programmed males showed however several hundreds of differentially expressed transcripts in white adipose tissue and liver. Interestingly, none of the transcripts reached the significance threshold for differential expression within the transcriptomic profile of brown adipose tissue between the two strains (Fig. 28). Selected transcripts of importance were validated by qPCR and summarized in Table 1. The effect of programming by maternal HSD feeding on change of transcriptomic profile was evident in both strains and all analysed tissues (Fig. 29). The analysis of canonical pathways, upstream regulators and mechanistic networks revealed mostly comparable results even when the overlap among the up- or downregulated transcripts by maternal HSD between the two strains was limited.

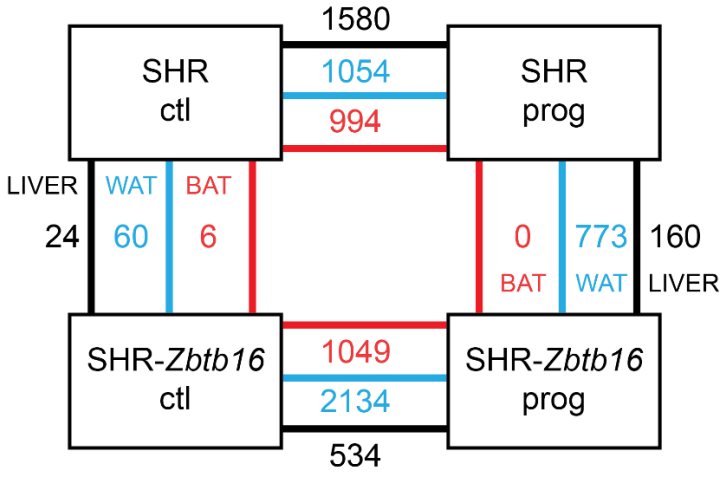


Fig. 28. Schematic depiction of transcriptome results comparison in adult male offspring of SHR and SHR-Zbtb16 rat strains. Numbers of significantly differentially expressed transcripts (FDR<0.05, >1.5 fold-change) between control (ctl) and maternally HSD programmed (prog) groups of both strains are shown for liver (black lines), white adipose tissue (WAT, blue lines) and brown adipose tissue (BAT, red lines).

Fig. 29. shows the result of the comparison of HSD programming effect for upstream regulators in the two strains across all tissues. The profile is very similar in the liver and brown adipose tissues of both strains, except for the distinctive pattern of white adipose tissue in SHR-Zbtb16.

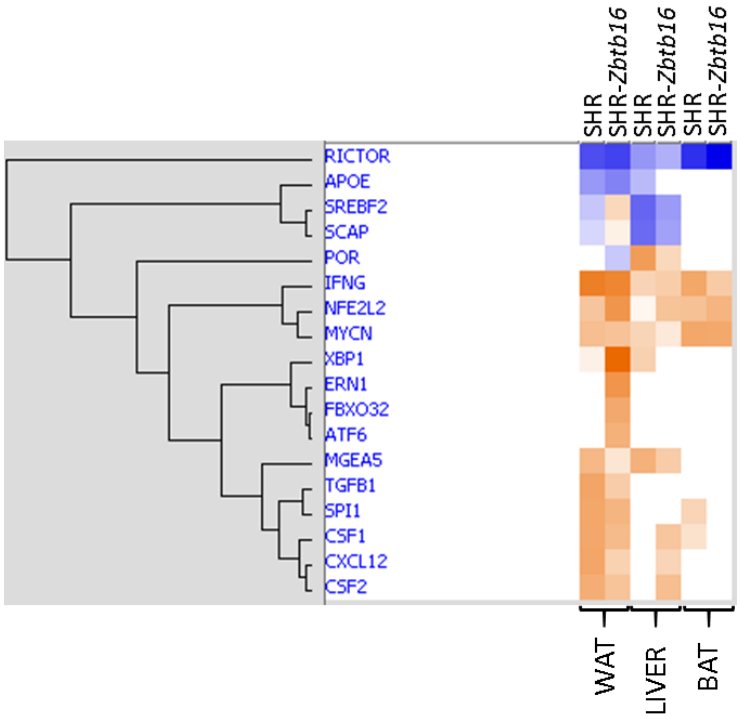


Fig. 29. Comparison of maternal HSD programming effect on activation (shades of orange) or inhibition (shades of blue) of upstream regulators in white adipose tissue (WAT), liver and brown adipose tissue (BAT) in male offspring of HSD- versus standard diet-fed rat dams of SHR and SHR-Zbtb16 rat strains. Hierarchical clustering and calculation of activation z-scores were performed using Ingenuity Pathways Analysis.

Fig. 30 depicts mechanistic network highlighting the key nodes with the highest score connecting the differentially expressed transcripts, their upstream regulators and downstream processes. The mechanistic network was derived in attempt to summarize the mechanism underlying observed metabolic shifts on the transcriptome level. The expression changes of 25 transcripts converged to three metabolic processes, which were perturbed by HSD-induced programming – metabolism of cholesterol, glucose and synthesis of cholesterol esters (fig network). This pattern was most evident in SHR rats, nonetheless we observed similar pattern in SHR-Zbtb16.

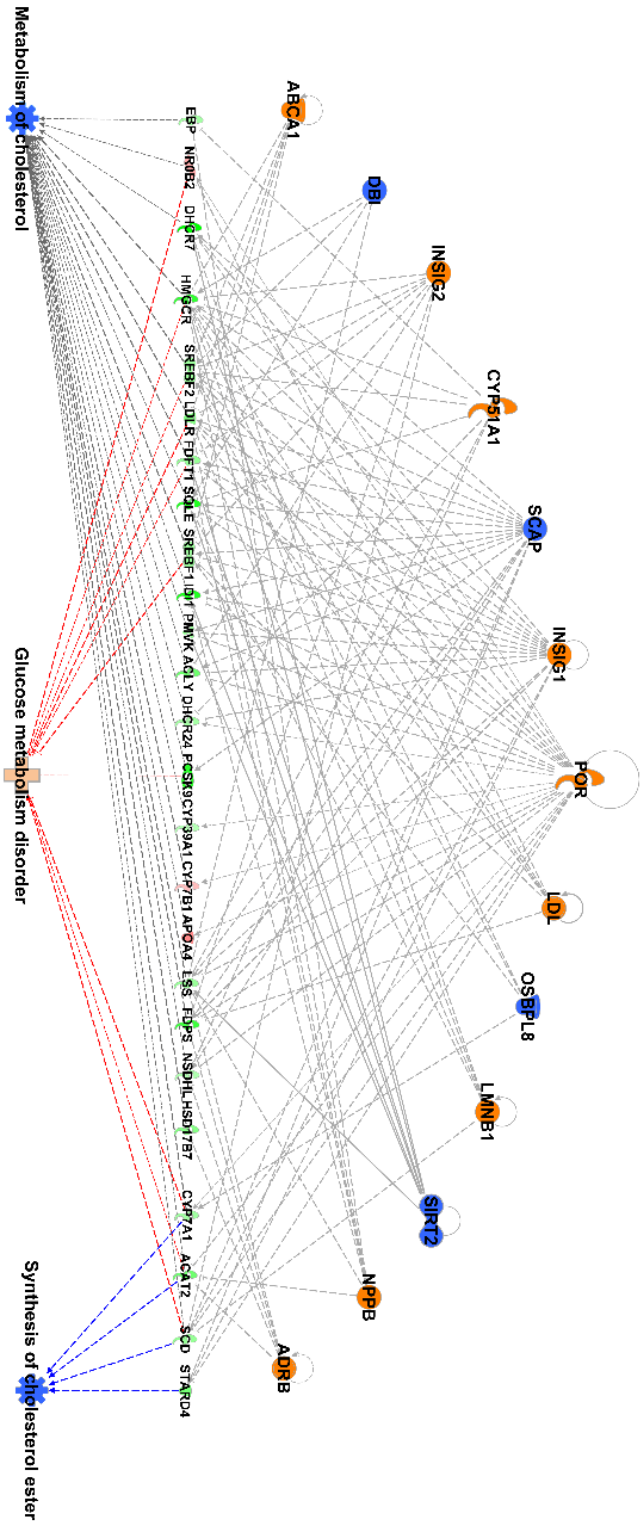


Fig. 30. Mechanistic network summarizing main effects of maternal HSD programming effect on activation (shades of orange) or inhibition (shades of blue) of upstream regulators in white adipose tissue, liver, and brown adipose tissue in male offspring of HSD- versus standard diet-fed rat dams of SHR and SHR-Zbtb16 rat strains. The programming effect on the expression of genes significantly differentially expressed in the above datasets is shown in shades of green (downregulation) or red (upregulation). Derivation of the network was performed using Ingenuity Pathways Analysis.

Gene symbol	Gene name	p (SHR prog vs. SHR ctl)	Fold change	p (SHR-Zbtb16 prog vs. SHR-Zbtb16 ctl)	Fold change
Brown adipose tissue					
Dio2	Iodothyronine deiodinase 2	3.8E-05	-2.8	4.1E-04	-2.3
Sod1	Superoxide dismutase 1	1.6E-07	1.6	2.4E-09	1.7
Cox8b	Cytochrome c oxidase subunit VIIIb	3.7E-10	1.9	1.5E-16	3.1
Hsd11b1	Hydroxysteroid 11-beta dehydrogenase 1	2.2E-05	2.0	n.s.	n.s.
White adipose tissue					
Slc2a4	Solute carrier family 2 member 4	4.9E-08	-3.0	1.6E-04	-1.9
Vegfb	Vascular endothelial growth factor B	3.7E-05	-2.2	9.9E-06	-2.3
Hsd11b1	Hydroxysteroid 11-beta dehydrogenase 1	3.7E-06	2.2	7.0E-04	1.7
Apoe	Apolipoprotein E	6.7E-09	3.6	4.1E-08	3.2
Liver					
Pcsk9	Proprotein convertase subtilisin/kexin type 9	3.5E-10	-4.5	7.7E-08	-3.3
Sqle	Squalene epoxidase	4.4E-10	-9.5	5.3E-06	-4.2
Srebf1	Sterol regulatory element binding transcription factor 1	1.1E-04	-2.1	1.0E-04	-2.1
Nr0b2	Nuclear receptor subfamily 0 group B member 2	3.6E-04	2.6	n.s.	n.s.
Acot1	Acyl-CoA thioesterase 1	6.6E-14	16.3	5.0E-11	8.9

Table 1. Offspring tissue-specific transcripts and their expression change in response to high-sucrose diet administration to rat dams. Transcripts validated by qPCR.

5.1.2.4.1. Liver tissue

Transcriptome profile of SHR male liver tissue differed in 1444 transcripts between controls and HSD programmed group. HSD programming in early development of SHR-Zbtb16 males changed the expression of 419 genes. The expression of 227 genes changed significantly in both strains as a result of maternal HSD programming, whereas 1217 transcripts were differentially expressed exclusively in SHR programmed males and 192 transcripts were differentially expressed exclusively in SHR-Zbtb16 programmed males. More distinct effect of HSD programming in liver tissue in SHR programmed males was a result of predominantly downregulated pathways of cholesterol biosynthesis including CYP51A1 (cytochrome p450 family 51 subfamily A member 1), DHCR7 (7-dehydrocholesterol reductase) and SQLE (squalene epoxidase). The effect of programming in liver tissue of SHR-Zbtb16 males was observed to be more subtle, determined by lesser downregulation of cholesterol biosynthesis pathway and altered pathway of mitochondrial dysfunction including cytochrome c oxidase subunit genes, cytochrome B and NADH dehydrogenase.

5.1.2.4.2. White adipose tissue

The transcriptomic analysis of white adipose tissue also revealed differences between strains in response to metabolic programming with HSD in early development. SHR programmed males showed significant differences in 935 transcripts compared to control, of which 479 were altered exclusively in SHR. White adipose tissue of programmed SHR-Zbtb16 males displayed a more pronounced response to HSD programming, as the expression of 2118 genes was significantly different compared to control SHR-Zbtb16 males, of which 1662 transcripts were unique for SHR-Zbtb16. The 456 transcripts common for both programmed groups of males were involved in EIF2 signalling and phagosome maturation pathways, which were both upregulated.

5.1.2.4.3. Brown adipose tissue

The expression changes in brown adipose tissue in response to HSD programming in early life were comparable between the two strains. SHR programmed males had 451 significant differentially expressed transcripts in brown fat compared to control SHR males, out

of which 206 were common with SHR-Zbtb16 programmed males' transcripts. SHR-Zbtb16 programmed males showed significant changes in 420 transcripts compared to control, out of which 214 were changed only in this strain. The pathways that were upregulated in brown adipose tissue of both strains were sirtuin signalling pathway, mTOR signalling, oxidative phosphorylation and mitochondrial dysfunction.

5.2. Chapter 2 – Study 2

5.2.1. Morphometry

Metabolic programming with HSD in combination with HSD feeding for 14 days increased relative weights of liver in SHR-Zbtb16 with no significant increase in SHR (Fig. 32). Absolute as well as relative weights of adrenals were also significantly higher in SHR-Zbtb16 re-exposed to HSD, with no similar effect in SHR males re-exposed to HSD (Fig. 33). Expected increase of fat mass after HSD feeding in adulthood manifested in absolute as well as relative weights of retroperitoneal fat pads and epididymal fat pads, with no significant changes in weights of interscapular brown fat tissue compared to HSD/HSD programmed animals fed STD (Fig. 34, 35). Muscle mass in m. soleus decreased in HSD-fed HSD/HSD programmed SHR-Zbtb16 males as opposed to no change in HSD-fed HSD/HSD programmed SHR males (Fig. 36).

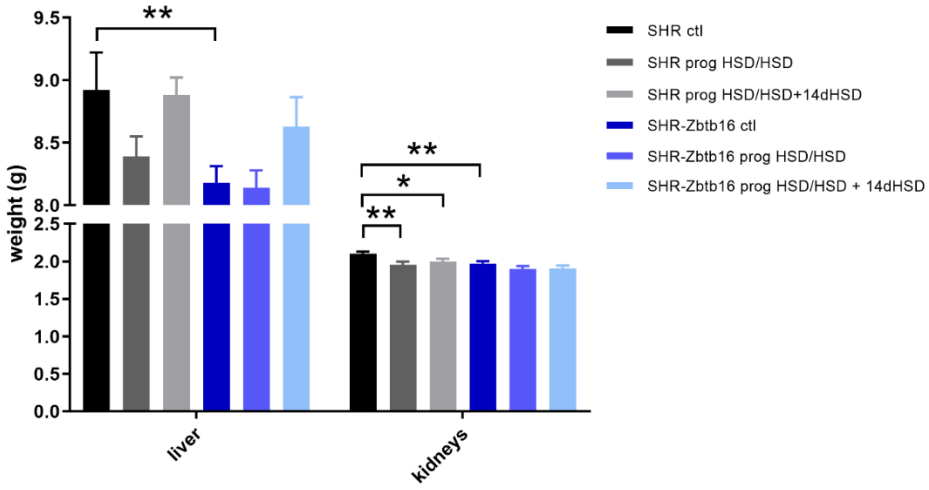


Fig. 31. Liver and kidney weight in adult F1 SHR control males (black bars), SHR males programmed with maternal HSD both in pregnancy and lactation (grey bars), SHR males programmed with maternal HSD both in

pregnancy and lactation re-exposed to HSD in adulthood (light grey bars), SHR-Zbtb16 control males (dark blue bars), SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation (blue bars) and SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation re-exposed to HSD in adulthood (light blue bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and MATERNAL DIET as major factors) Fisher's test of pairwise group comparison (control vs programmed vs re-exposed males) within each strain are indicated as follows: * p<0.05, ** p<0.01.

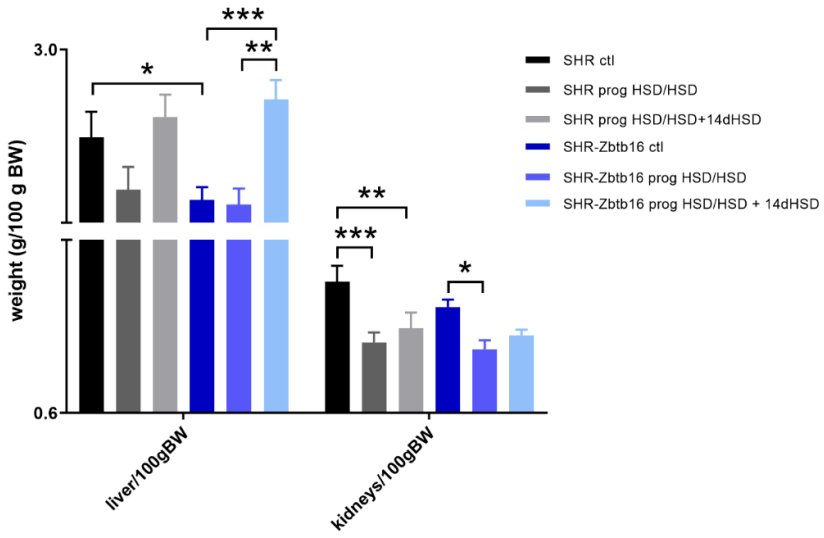


Fig. 32. Liver and kidney weight per 100g of body weight in adult F1 SHR control males (black bars), SHR males programmed with maternal HSD both in pregnancy and lactation (grey bars), SHR males programmed with maternal HSD both in pregnancy and lactation re-exposed to HSD in adulthood (light grey bars), SHR-Zbtb16 control males (dark blue bars), SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation (blue bars) and SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation re-exposed to HSD in adulthood (light blue bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and MATERNAL DIET as major factors) Fisher's test of pairwise group comparison (control vs programmed vs re-exposed males) within each strain are indicated as follows: * p<0.05, ** p<0.01, *** p<0.001.

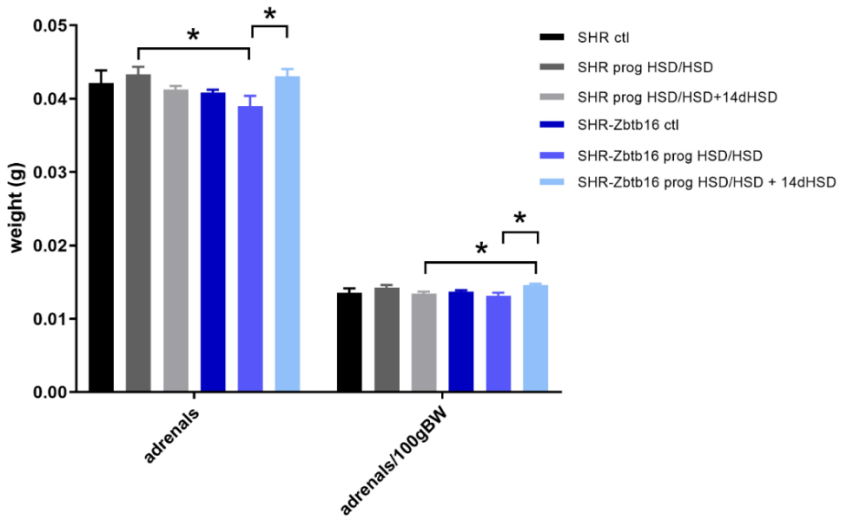


Fig. 33. Adrenal weight in adult F1 SHR control males (black bars), SHR males programmed with maternal HSD both in pregnancy and lactation (grey bars), SHR males programmed with maternal HSD both in pregnancy and lactation re-exposed to HSD in adulthood (light grey bars), SHR-Zbtb16 control males (dark blue bars), SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation (blue bars) and SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation re-exposed to HSD in adulthood (light blue bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and MATERNAL DIET as major factors) Fisher's test of pairwise group comparison (control vs programmed vs re-exposed males) within each strain are indicated as follows: * p<0.05.

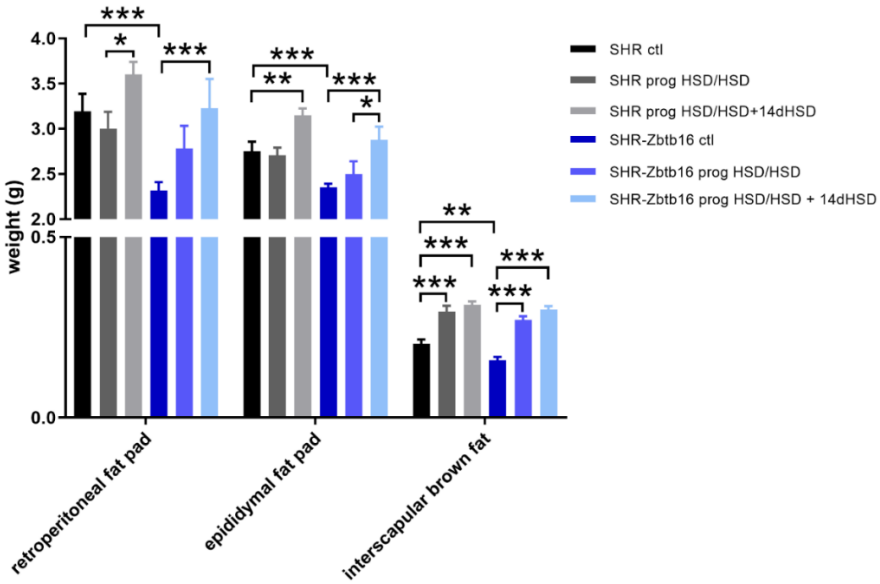


Fig. 34. Retroperitoneal fat pad, epididymal fat pad and interscapular brown fat weight in adult F1 SHR control males (black bars), SHR males programmed with maternal HSD both in pregnancy and lactation (grey bars), SHR

males programmed with maternal HSD both in pregnancy and lactation re-exposed to HSD in adulthood (light grey bars), SHR-Zbtb16 control males (dark blue bars), SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation (blue bars) and SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation re-exposed to HSD in adulthood (light blue bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and MATERNAL DIET as major factors) Fisher's test of pairwise group comparison (control vs programmed vs re-exposed males) within each strain are indicated as follows: * p<0.05, ** p<0.01, *** p<0.001.

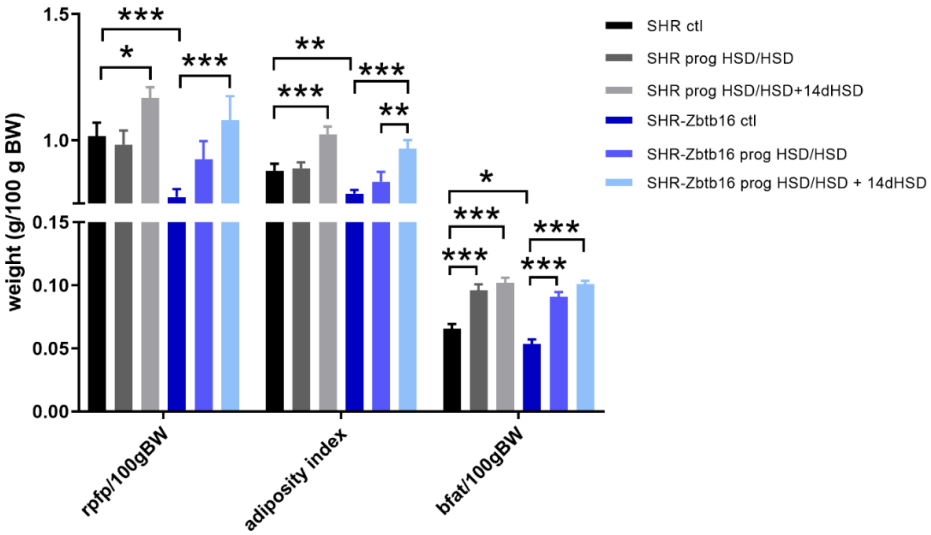


Fig. 35. Retroperitoneal fat pad, epididymal fat pad (adiposity index) and interscapular brown fat weight per 100 g of body weight in adult F1 SHR control males (black bars), SHR males programmed with maternal HSD both in pregnancy and lactation (grey bars), SHR males programmed with maternal HSD both in pregnancy and lactation re-exposed to HSD in adulthood (light grey bars), SHR-Zbtb16 control males (dark blue bars), SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation (blue bars) and SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation re-exposed to HSD in adulthood (light blue bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and MATERNAL DIET as major factors) Fisher's test of pairwise group comparison (control vs programmed vs re-exposed males) within each strain are indicated as follows: * p<0.05, ** p<0.01, *** p<0.001.

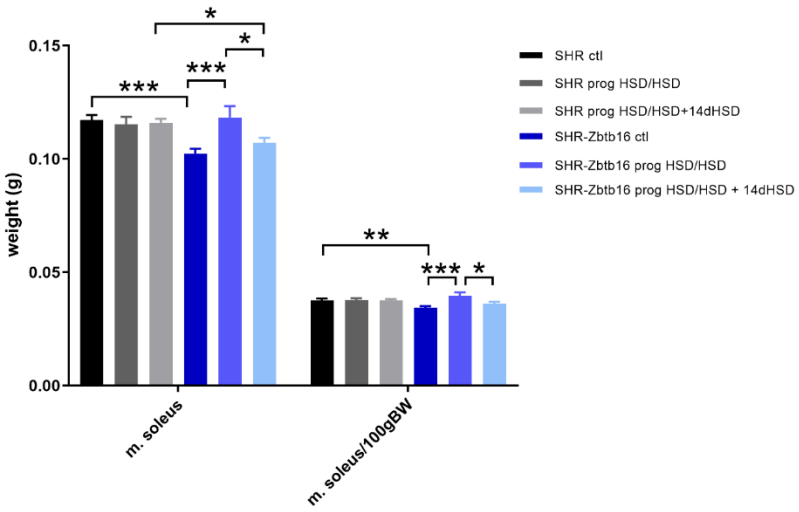


Fig. 36. Hind leg skeletal muscle (musculus soleus) weight in adult F1 SHR control males (black bars), SHR males programmed with maternal HSD both in pregnancy and lactation (grey bars), SHR males programmed with maternal HSD both in pregnancy and lactation re-exposed to HSD in adulthood (light grey bars), SHR-Zbtb16 control males (dark blue bars), SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation (blue bars) and SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation re-exposed to HSD in adulthood (light blue bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and MATERNAL DIET as major factors) Fisher's test of pairwise group comparison (control vs programmed vs re-exposed males) within each strain are indicated as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

5.2.2. Glucose tolerance and insulin levels

During oral glucose tolerance test, HSD re-exposed programmed SHR-Zbtb16 males showed worsened glucose tolerance in comparison with respective SHR group. Compared to the control, HSD re-exposed programmed SHR males had significantly lower fasting glycaemia (Fig. 37), similarly to only programmed SHR males fed STD. In comparison to HSD/HSD programmed SHR-Zbtb16 males, which were not exposed to HSD in adulthood, the blood glucose levels 30, 60 and 90 minutes after the load were significantly higher in re-exposed HSD/HSD programmed SHR-Zbtbt16 males and therefore their glucose tolerance had decreased (Fig. 38). Re-exposed SHR programmed males show no significant differences in their blood glucose levels compared to only programmed SHR males except for 3 hours after the glycaemic load, when re-exposed SHR males had higher glycaemia (Fig. 38). Area under the glycaemic curve increased significantly in re-exposed HSD/HSD programmed SHR-Zbtb16 males compared to only programmed SHR-Zbtb16 males and also control SHR-Zbtb16 males,

with no similar effect in SHR (Fig. 39). We identified STRAIN*DIET interactions for AUCres180 (p=0.009).

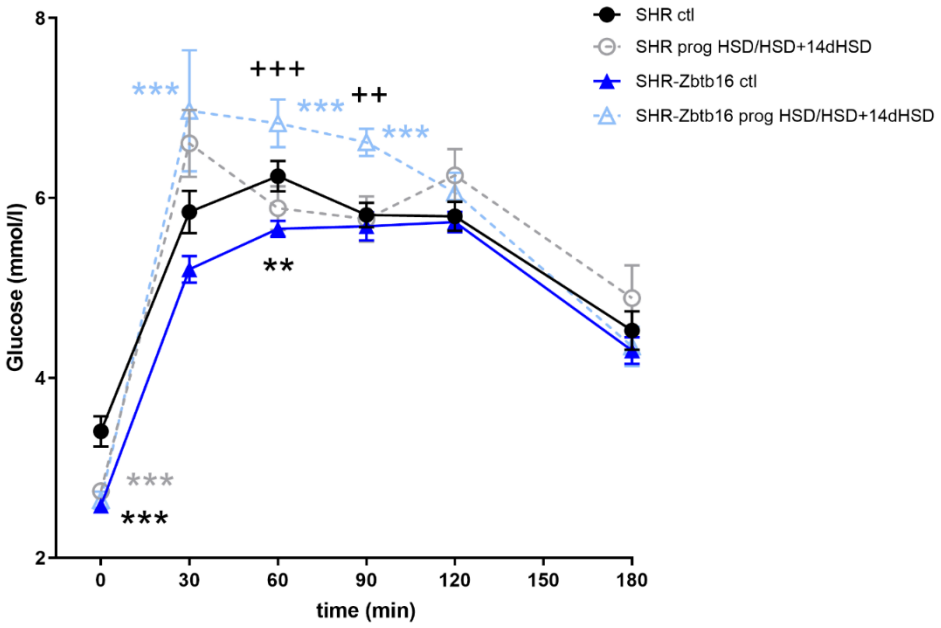


Fig. 37. The oral glucose tolerance test (OGTT). The course of glycaemic curves in adult F1 SHR control males (black circles, full line), SHR males programmed with maternal HSD both in pregnancy and lactation re-exposed to HSD in adulthood (light grey empty circles, dashed line), SHR-Zbtb16 control males (dark blue triangles, full line) and SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation re-exposed to HSD in adulthood (light blue empty triangles, dashed line). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and MATERNAL DIET as major factors) Fisher's test of pairwise group comparison (control vs programmed vs re-exposed males) within each strain are indicated as follows: ** p<0.01, *** p<0.001. Strain differences between SHR control males and SHR-Zbtb16 control males are represented by black asterisks (*), t=0 min ***, t=60 min **; SHR HSD/HSD programmed re-exposed males and SHR-Zbtb16 HSD/HSD programmed re-exposed males are represented by black plus signs (+), t=60 min ***, t=90 min **. Effect of programming and re-exposure in SHR males is represented by grey asterisks (*) – significant differences between control and programmed re-exposed males, t=0 min ***; in SHR-Zbtb16 males is represented by light blue asterisks (*) - significant differences between control and programmed re-exposed males, t=30 min ***, t= 60 min ***, t=90 min ***.

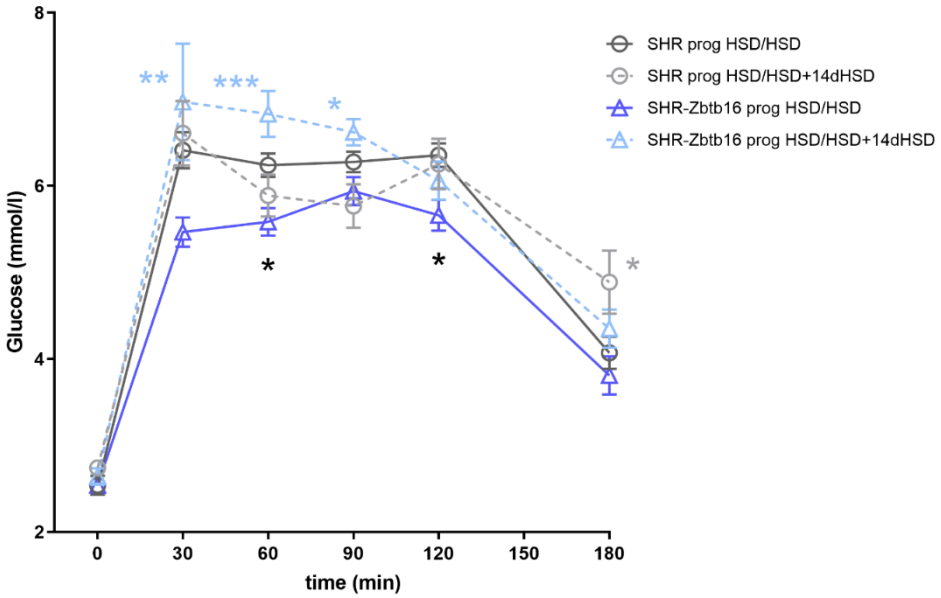


Fig. 38. The oral glucose tolerance test (OGTT). The course of glycaemic curves in adult F1 SHR males programmed with maternal HSD both in pregnancy and lactation (grey empty circles, full line), SHR males programmed with maternal HSD both in pregnancy and lactation re-exposed to HSD in adulthood (light grey empty circles, dashed line), SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation (blue empty triangles, full line) and SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation re-exposed to HSD in adulthood (light blue empty triangles, dashed line). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and MATERNAL DIET as major factors) Fisher's test of pairwise group comparison (control vs programmed vs re-exposed males) within each strain are indicated as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Strain differences between SHR HSD/HSD programmed males and SHR-Zbtb16 HSD/HSD programmed males are represented by black asterisks (*), t=60 min *, t=120 min *. Effect of re-exposure in SHR males is represented by grey asterisks (*) – significant differences between programmed and programmed re-exposed males, t=180 min *; in SHR-Zbtb16 males is represented by light blue asterisks (*) - significant differences between programmed and programmed re-exposed males, t=30 min **, t= 60 min ***, t=90 min *.

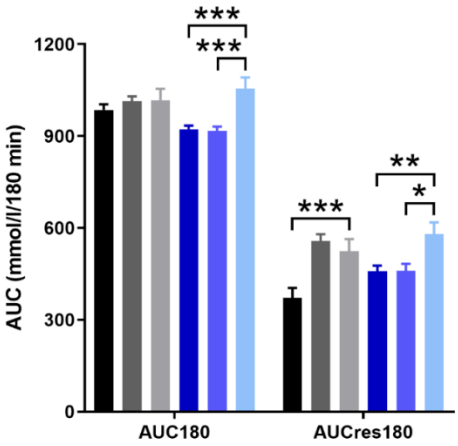


Fig. 39. Area under the curve. Area under the glycaemic curve values calculated from the course of glycaemic curves from 0 to 180 (AUC180) and value of the residual area under the curve (AUCres180). SHR control males (black bars), SHR males programmed with maternal HSD both in pregnancy and lactation (grey bars), SHR males programmed with maternal HSD both in pregnancy and lactation re-exposed to HSD in adulthood (light grey bars), SHR-Zbtb16 control males (dark blue bars), SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation (blue bars) and SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation re-exposed to HSD in adulthood (light blue bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and MATERNAL DIET as major factors) Fisher's test of pairwise group comparison (control vs programmed vs re-exposed males) within each strain are indicated as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

HSD/HSD programmed re-exposed males of both strains showed the highest fasting insulin concentrations of all their respective groups (Fig. 40). Metabolic programming in HSD/HSD regime combined with 14 days re-exposure to HSD increased fasting insulin levels in SHR-Zbtb16 significantly, in SHR the levels only matched the controls.

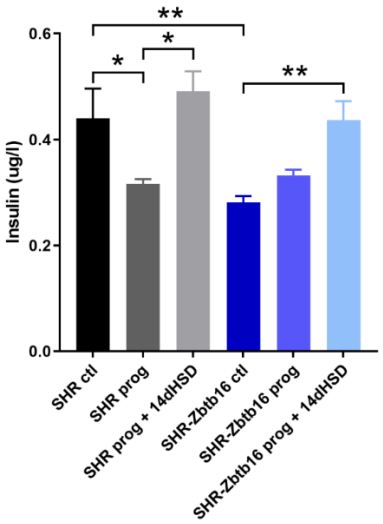
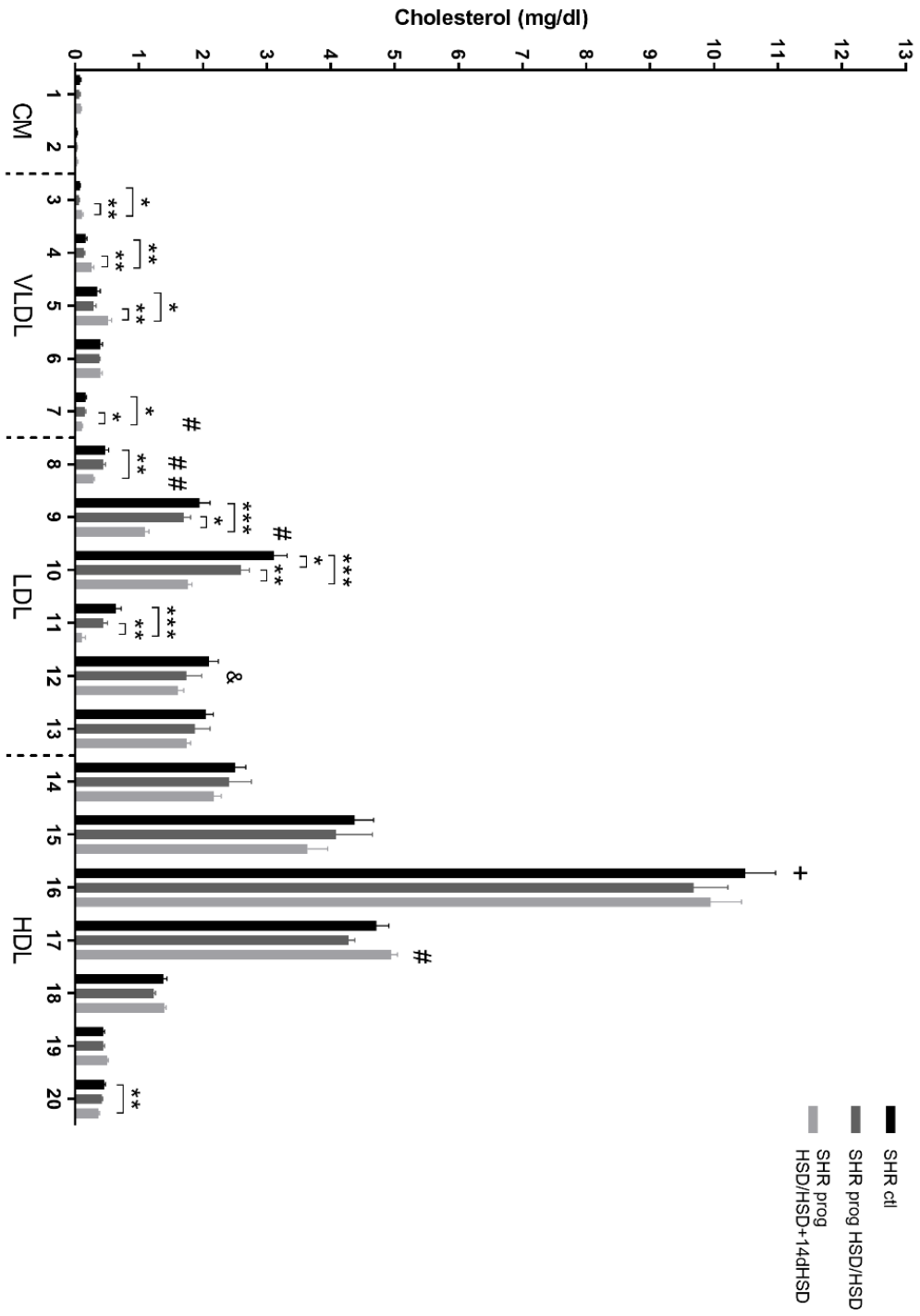


Fig. 40. Fasting insulin concentrations in SHR control males (black bars), SHR males programmed with maternal HSD both in pregnancy and lactation (grey bars), SHR males programmed with maternal HSD both in pregnancy and lactation re-exposed to HSD in adulthood (light grey bars), SHR-Zbtb16 control males (dark blue bars), SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation (blue bars) and SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation re-exposed to HSD in adulthood (light blue bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and MATERNAL DIET as major factors) Fisher's test of pairwise group comparison (control vs programmed vs re-exposed males) within each strain are indicated as follows: * $p<0.05$, ** $p<0.01$.

5.2.3. Lipid profile

Cholesterol profile shifts were similar in both strains for their respective groups. In re-exposed programmed SHR-Zbtb16 males, small VLDL, large and medium LDL and medium HDL fractions were increased compared to re-exposed programmed SHR males (Fig. 41). The cholesterol content in major lipoprotein class of chylomicrons and very low density lipoproteins was increased in re-exposed programmed SHR-Zbtb16 males only, whereas low density lipoprotein contents were decreased in both re-exposed groups compared to control groups (Fig. 42). Not only did very low density lipoproteins increased in content, their particle size was also significantly bigger in both re-exposed groups compared to their controls (Fig. 43). The particle size of high density lipoproteins however decreased only in re-exposed SHR-Zbtb16 males.


Cholesterol (mg/dl)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
CM
VLDL
LDL
HDL
SHR ctl
SHR prog HSD/HSD
SHR prog HSD/HSD+14dHSD

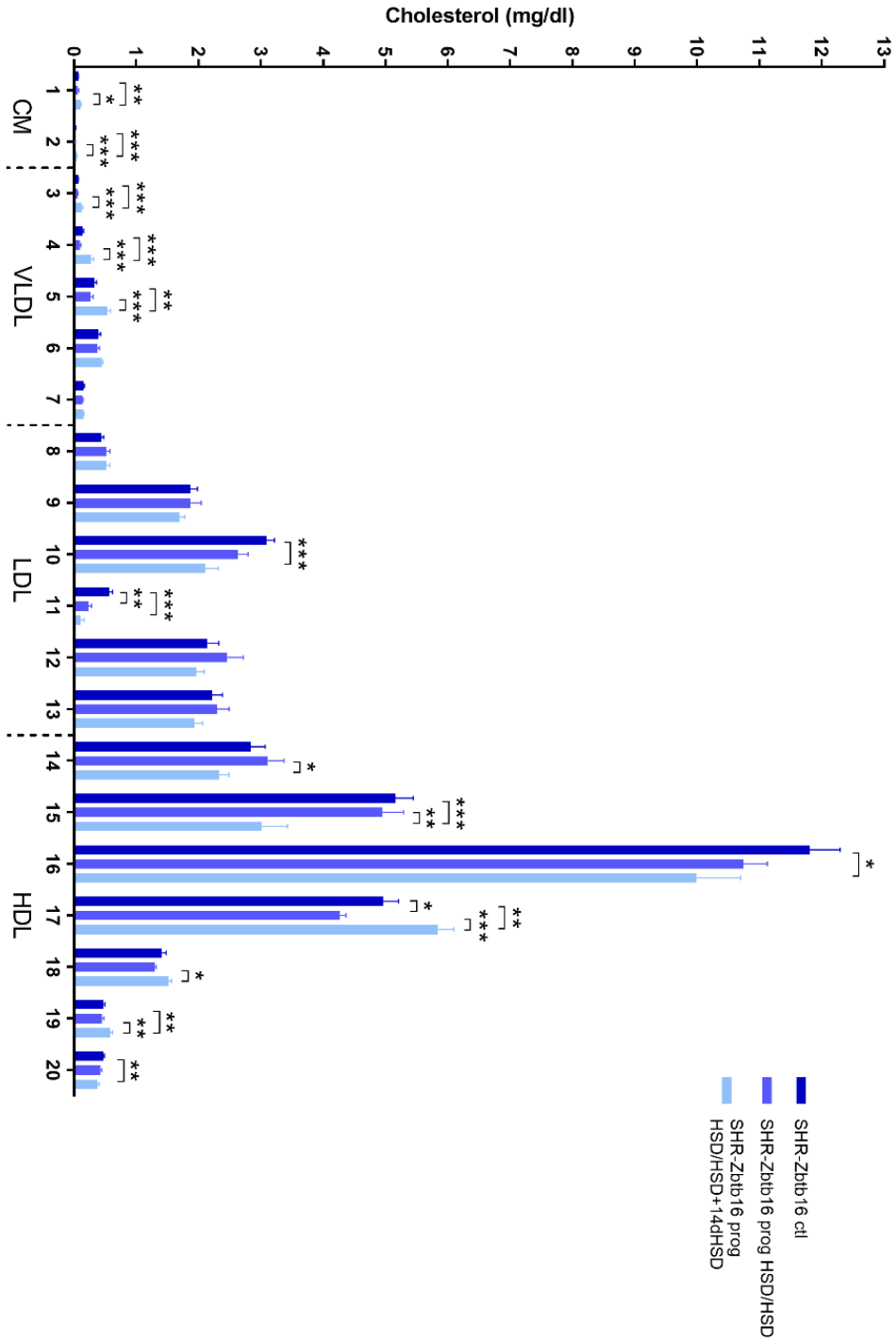


Fig. 41. Cholesterol profile. The cholesterol (C) content in 20 lipoprotein subfractions in SHR control males (black bars), SHR males programmed with maternal HSD both in pregnancy and lactation (grey bars), SHR males programmed with maternal HSD both in pregnancy and lactation re-exposed to HSD in adulthood (light grey bars), SHR-Zbtb16 control males (dark blue bars), SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation (blue bars) and SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation re-exposed to HSD in adulthood (light blue bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and MATERNAL DIET as major factors) Fisher's test of pairwise group comparison (control vs programmed vs re-exposed males) within each strain are

indicated as follows: * p<0.05, ** p<0.01, *** p<0.001. Strain differences between SHR control males and SHR-Zbtb16 control males are represented by black plus sign (+), C 16 *; SHR HSD/HSD programmed males and SHR-Zbtb16 HSD/HSD programmed males are represented by black ampersand (&), C 12 *; SHR HSD/HSD programmed and re-exposed males and SHR-Zbtb16 HSD/HSD programmed and re-exposed males are represented by black hashtag (#), C 7 *, C 8 **, C 9 *, C 17 *. The allocation of individual lipoprotein subfractions to major lipoprotein classes is shown in order of particle's decreasing size from left to right. CM-chylomicron, VLDL-very low density lipoprotein, LDL-low density lipoprotein, HDL-high density lipoprotein.

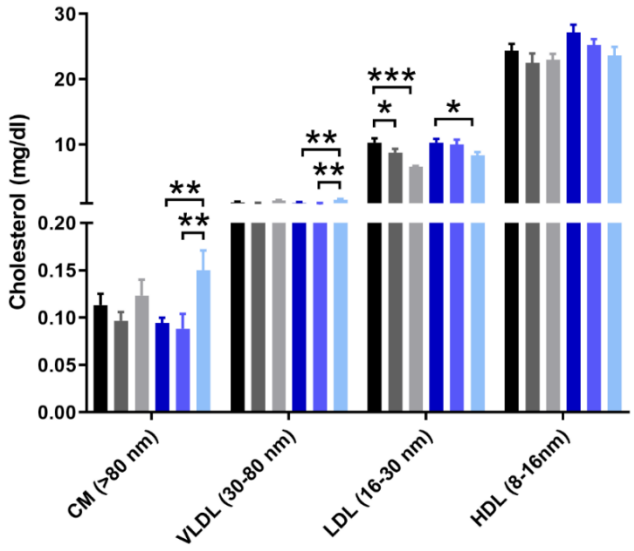


Fig. 42. Cholesterol content. The cholesterol (C) content in major lipoprotein classes in SHR control males (black bars), SHR males programmed with maternal HSD both in pregnancy and lactation (grey bars), SHR males programmed with maternal HSD both in pregnancy and lactation re-exposed to HSD in adulthood (light grey bars), SHR-Zbtb16 control males (dark blue bars), SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation (blue bars) and SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation re-exposed to HSD in adulthood (light blue bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and MATERNAL DIET as major factors) Fisher's test of pairwise group comparison (control vs programmed vs re-exposed males) within each strain are indicated as follows: * p<0.05, ** p<0.01, *** p<0.001. CM-chylomicron, VLDL-very low density lipoprotein, LDL-low density lipoprotein, HDL-high density lipoprotein.

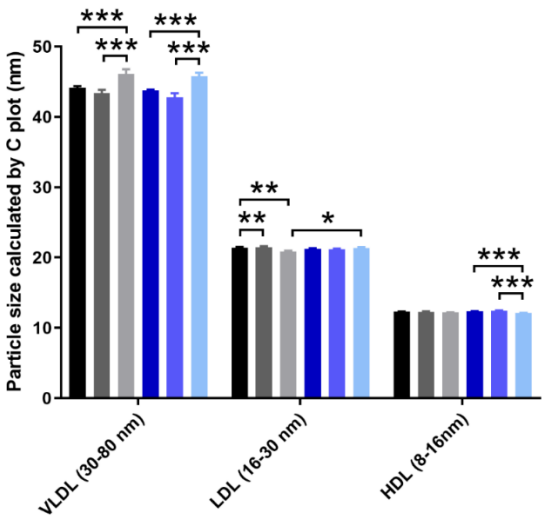
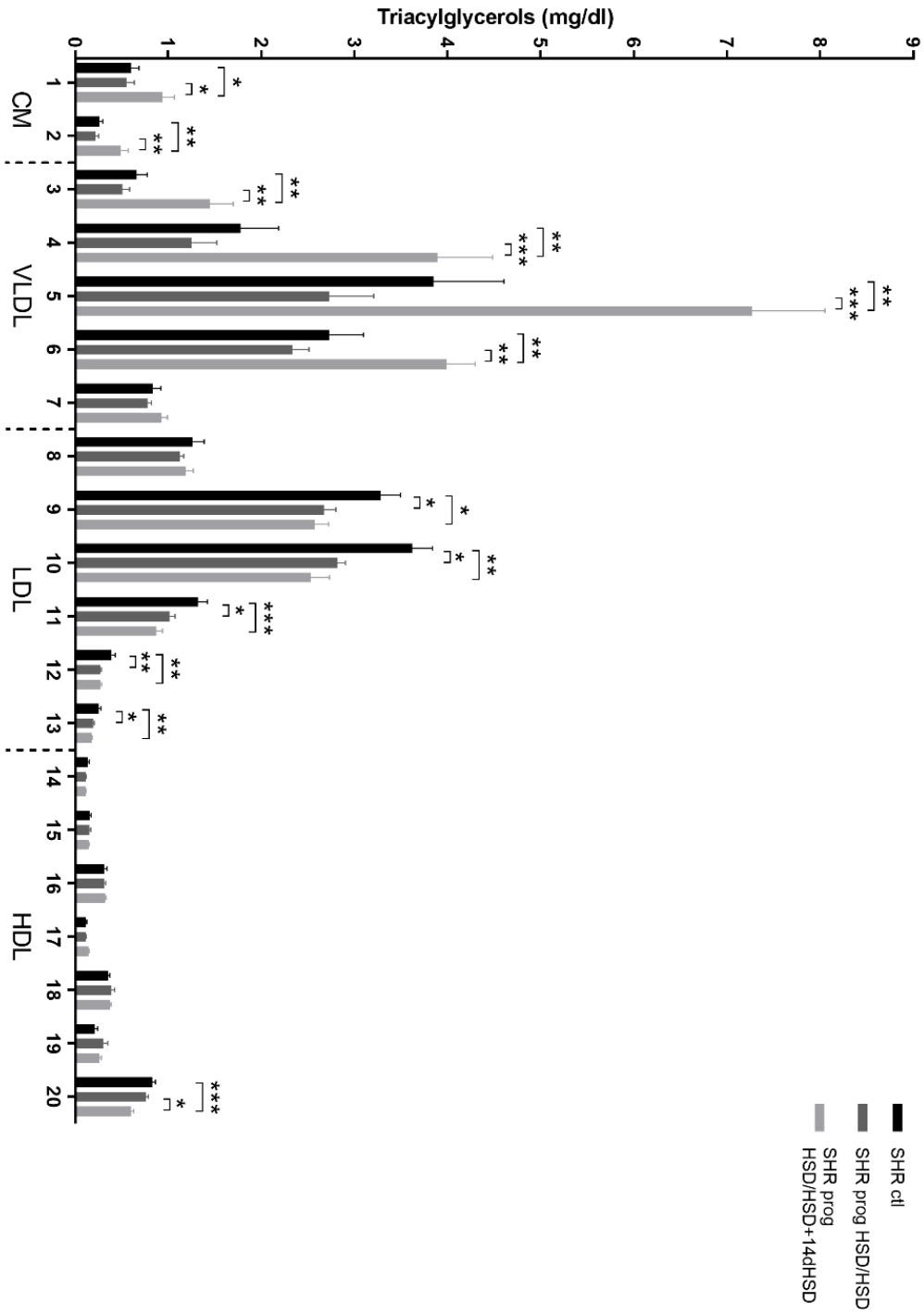


Fig. 43. Major lipoprotein classes particle size calculated by C plot in SHR control males (black bars), SHR males programmed with maternal HSD both in pregnancy and lactation (grey bars), SHR males programmed with maternal HSD both in pregnancy and lactation re-exposed to HSD in adulthood (light grey bars), SHR-Zbtb16 control males (dark blue bars), SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation (blue bars) and SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation re-exposed to HSD in adulthood (light blue bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and MATERNAL DIET as major factors) Fisher's test of pairwise group comparison (control vs programmed vs re-exposed males) within each strain are indicated as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. VLDL-very low density lipoprotein, LDL-low density lipoprotein, HDL-high density lipoprotein.

The analysis of triacylglycerols distribution showed no significant differences between strains in their respective groups (Fig. 44). The triacylglycerol content in major lipoprotein classes increased in chylomicrons and very low density lipoproteins classes and decreased in low density lipoproteins class in both groups of re-exposed males (Fig. 45). The particle size of very low density lipoprotein particles concurrently increased as well, however low density lipoprotein particles increased in size in both re-exposed groups (Fig. 46). Only in re-exposed SHR males the high density lipoprotein particle size increased.


Triacylglycerols (mg/dl)
0
1
2
3
4
5
6
7
8
9
CM
VLDL
LDL
HDL
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
SHR ctl
SHR prog HSD/HSD
SHR prog HSD/HSD+14dHSD

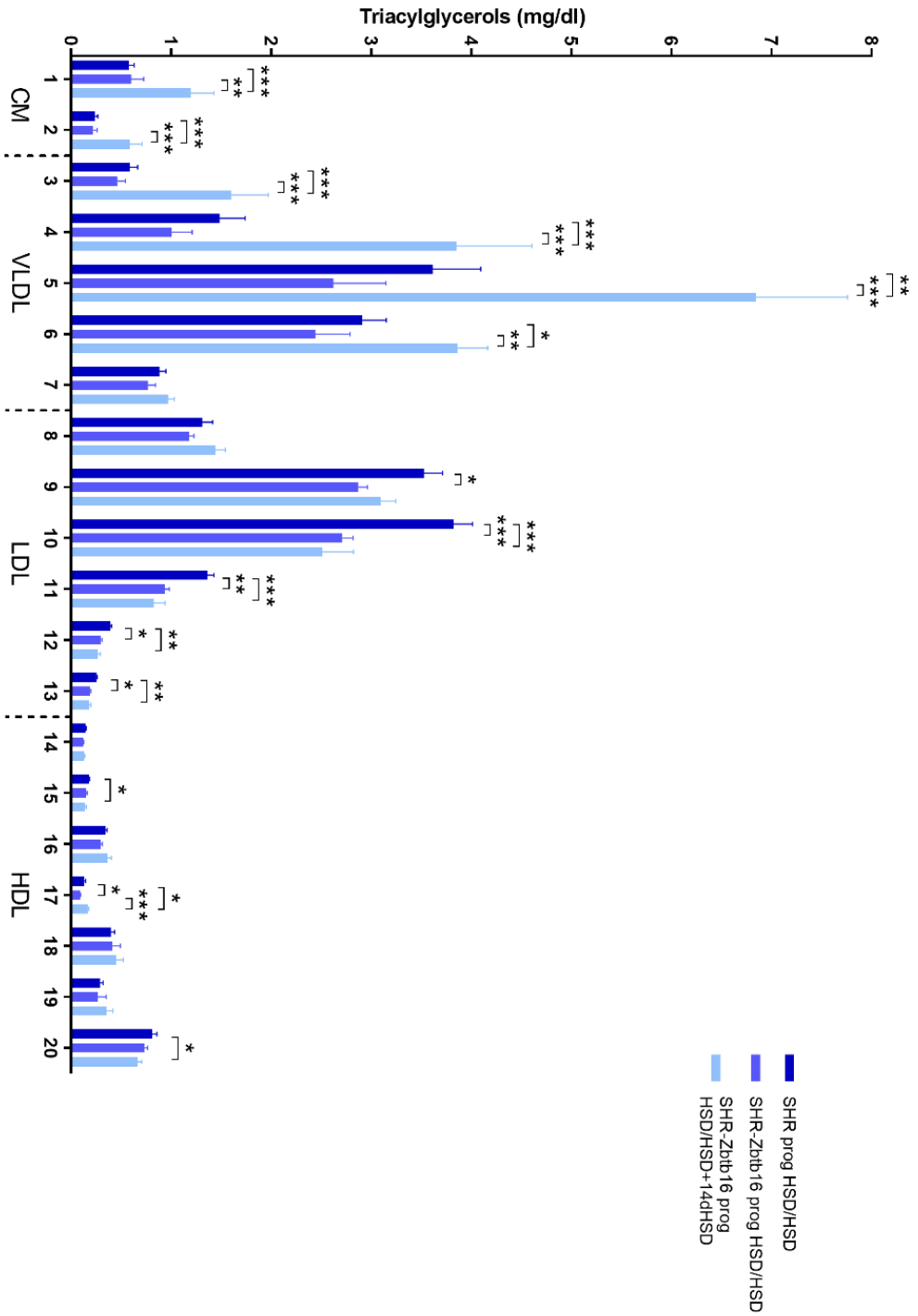


Fig. 44. Triacylglycerol profile. The triacylglycerol (TG) content in 20 lipoprotein subfractions in SHR control males (black bars), SHR males programmed with maternal HSD both in pregnancy and lactation (grey bars), SHR males programmed with maternal HSD both in pregnancy and lactation re-exposed to HSD in adulthood (light grey bars), SHR-Zbtb16 control males (dark blue bars), SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation (blue bars) and SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation re-exposed to HSD in adulthood (light blue bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and MATERNAL DIET as major factors) Fisher's test of pairwise group comparison (control vs programmed vs re-exposed males) within each strain are

indicated as follows: * p<0.05, ** p<0.01, *** p<0.001. The allocation of individual lipoprotein subfractions to major lipoprotein classes is shown in order of particle's decreasing size from left to right. CM-chylomicron, VLDL-very low density lipoprotein, LDL-low density lipoprotein, HDL-high density lipoprotein.

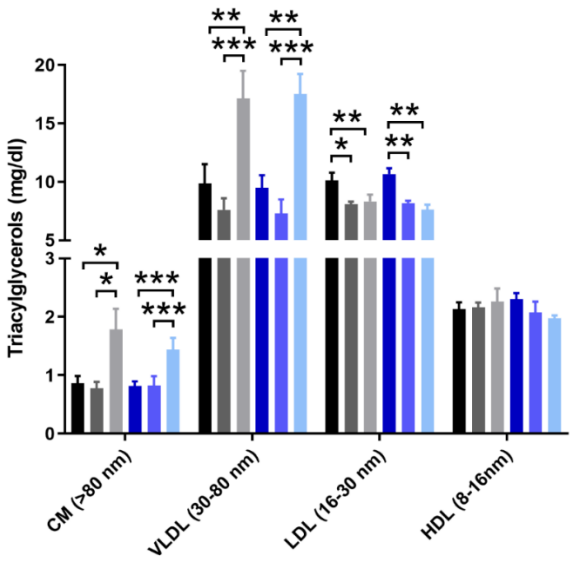


Fig. 45. Triacylglycerol content. The triacylglycerol (TG) content in major lipoprotein classes in SHR control males (black bars), SHR males programmed with maternal HSD both in pregnancy and lactation (grey bars), SHR males programmed with maternal HSD both in pregnancy and lactation re-exposed to HSD in adulthood (light grey bars), SHR-Zbtb16 control males (dark blue bars), SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation (blue bars) and SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation re-exposed to HSD in adulthood (light blue bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and MATERNAL DIET as major factors) Fisher's test of pairwise group comparison (control vs programmed vs re-exposed males) within each strain are indicated as follows: * p<0.05, ** p<0.01, *** p<0.001. CM-chylomicron, VLDL-very low density lipoprotein, LDL-low density lipoprotein, HDL-high density lipoprotein.

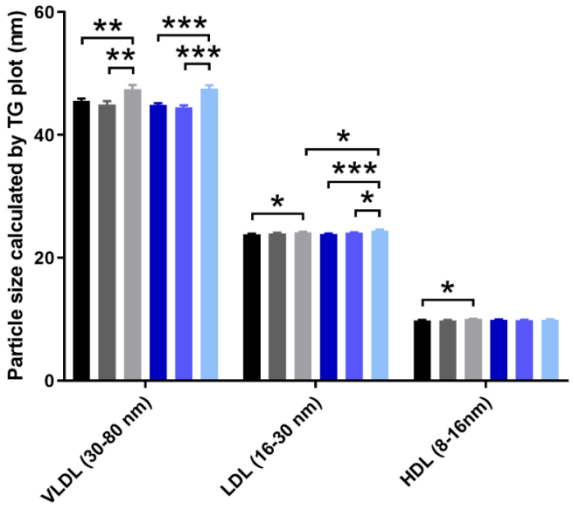


Fig. 46. Major lipoprotein classes particle size calculated by TG plot in SHR control males (black bars), SHR males programmed with maternal HSD both in pregnancy and lactation (grey bars), SHR males programmed with maternal HSD both in pregnancy and lactation re-exposed to HSD in adulthood (light grey bars), SHR-Zbtb16 control males (dark blue bars), SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation (blue bars) and SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation re-exposed to HSD in adulthood (light blue bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and MATERNAL DIET as major factors) Fisher's test of pairwise group comparison (control vs programmed vs re-exposed males) within each strain are indicated as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. VLDL-very low density lipoprotein, LDL-low density lipoprotein, HDL-high density lipoprotein.

Re-exposed programmed SHR-Zbtb16 group of males showed the highest levels of free glycerol in the serum (Fig. 47). The levels of glycerol were significantly higher than in HSD-programmed SHR-Zbtb16 males and control SHR-Zbtb16 males. Most importantly, levels of free glycerol differed significantly also with re-exposed programmed SHR males (increase by 15% in HSD-programmed SHR males re-exposed to HSD vs. by 46% in HSD-programmed SHR-Zbtb16 males re-exposed to HSD).

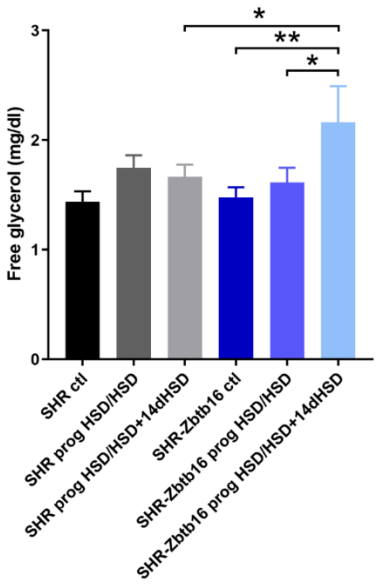


Fig. 47. Free glycerol serum concentrations in SHR control males (black bars), SHR males programmed with maternal HSD both in pregnancy and lactation (grey bars), SHR males programmed with maternal HSD both in pregnancy and lactation re-exposed to HSD in adulthood (light grey bars), SHR-Zbtb16 control males (dark blue bars), SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation (blue bars) and SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation re-exposed to HSD in adulthood (light blue bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and MATERNAL DIET as major factors) Fisher's test of pairwise group comparison (control vs programmed vs re-exposed males) within each strain are indicated as follows: * $p<0.05$, ** $p<0.01$.

5.3. Chapter 3 – Study 3

5.3.1. Mothers

5.3.1.1. Body weight and diet consumption

Body weight measurements comparison showed significantly lower weights of F1 HSD/HSD programmed SHR-Zbtb16 females at the same age as not programmed STD/STD SHR-Zbtb16 females (Fig. 48). The disparity between programmed and not programmed female body weights in adulthood disappeared in the pregnancy period. This effect transferred into post-delivery period of breastfeeding, when we did not record any significant differences in body weight between these groups. F1 HSD/HSD programmed SHR-Zbtb16 females were significantly lighter than F1 HSD/HSD programmed SHR females in the same period.

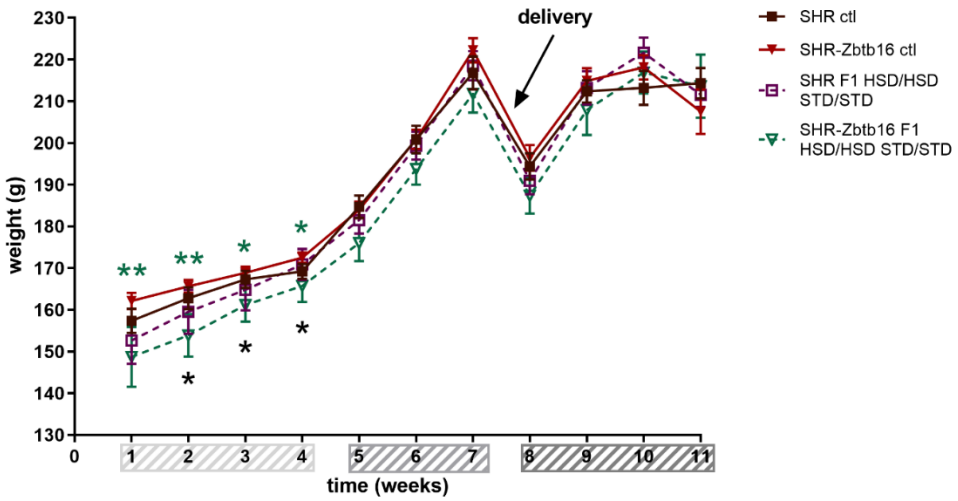


Fig. 48. Body weight measurements of F0 and F1 SHR and SHR-*Zbtb16* adult female rats from 16 weeks of age (weeks 1-4), in gravidity (weeks 5-7) and lactation period (weeks 8-11), F0 SHR control females (dark brown squares, full line), F1 SHR females HSD/HSD programmed (purple empty squares, dashed line), F0 SHR-*Zbtb16* control females (dark red triangles, full line), F1 SHR-*Zbtb16* females HSD/HSD programmed (dark teal empty triangles, dashed line). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and MATERNAL DIET as major factors) Fisher's test of pairwise group comparison within each strain are indicated as follows: * $p<0.05$, ** $p<0.01$. Strain differences between F1 HSD/HSD programmed SHR and SHR-Zbtb16 females are represented by black asterisks (*), week 2 *, week 3 *, week 4 *. Effects of programming in SHR-Zbtb16 females are represented by dark teal asterisks (*) - significant differences between F0 not programmed and F1 HSD/HSD programmed females, week 1 **, week 2 **, week 3 *, week 4 *.

F1 HSD/HSD programmed females of both strains significantly increased their diet consumption in pregnancy a thus increased their caloric intake (Fig. 49). However, in breastfeeding period, F1 HSD/HSD programmed SHR-Zbtb16 females showed significantly decreased diet intake compared to not programmed STD/STD females in the same period (Fig. 50).

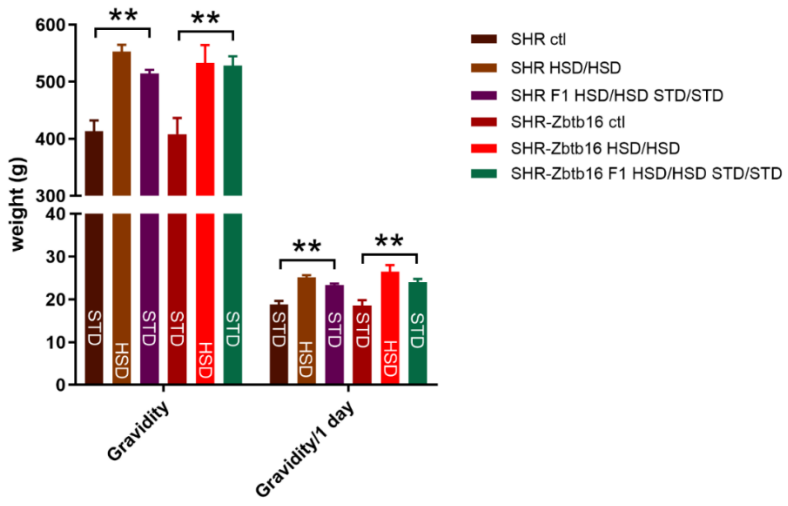


Fig. 49. Diet consumption in gravidity of F0 and F1 SHR and SHR-*Zbtb16* female rats, F0 SHR control females (dark brown bars), F0 SHR females fed HSD during pregnancy (light brown bars), F1 SHR females HSD/HSD programmed (purple bars), F0 SHR-*Zbtb16* control females (dark red bars), F0 SHR-*Zbtb16* females fed HSD during pregnancy (red bars) and F1 SHR-*Zbtb16* females HSD/HSD programmed (dark teal bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and MATERNAL DIET as major factors) Fisher's test of pairwise group comparison within each strain are indicated as follows: ** $p<0.01$.

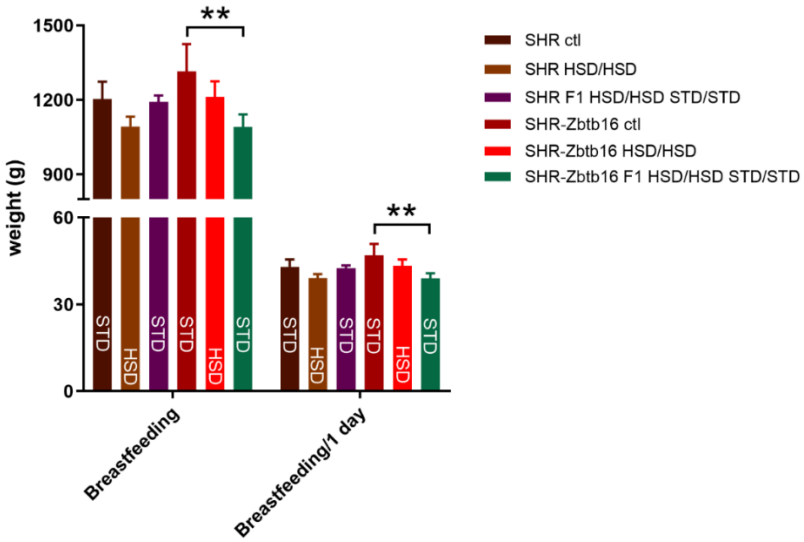


Fig. 50. Diet consumption in breastfeeding of F0 and F1 SHR and SHR-*Zbtb16* female rats. F0 SHR control females (dark brown bars), F0 SHR females fed HSD during pregnancy (light brown bars), F1 SHR females HSD/HSD programmed (purple bars), F0 SHR-*Zbtb16* control females (dark red bars), F0 SHR-*Zbtb16* females fed HSD during pregnancy (red bars) and F1 SHR-*Zbtb16* females HSD/HSD programmed (dark teal bars). Data

are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and MATERNAL DIET as major factors) Fisher's test of pairwise group comparison within each strain are indicated as follows: ** p<0.01.

5.3.1.2. Metabolic profile

Prior to pregnancy, the fasting glycaemia of F1 HSD/HSD programmed females of both strains was significantly elevated compared to controls (Fig. 51). This resulted in smaller area under the curve in F1 HSD/HSD programmed females (Fig. 52).

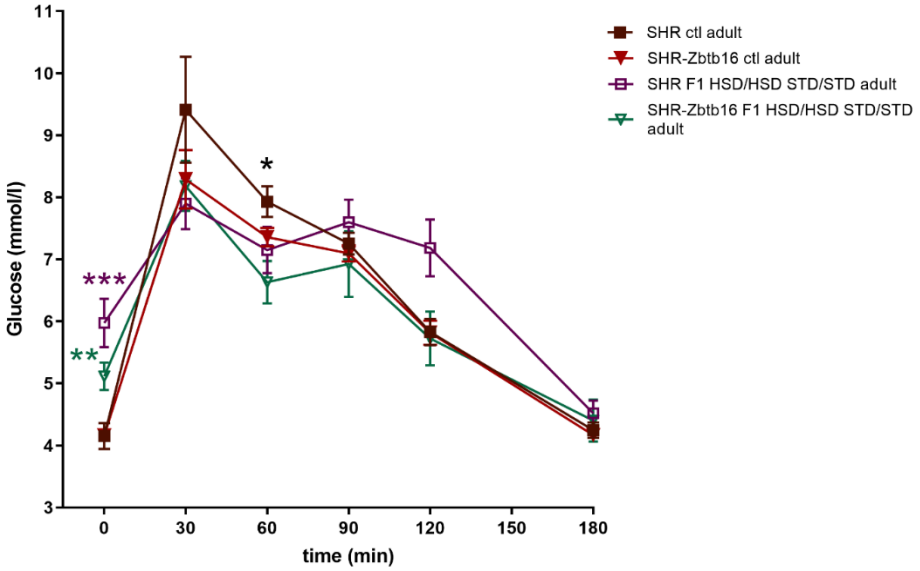


Fig. 51. The oral glucose tolerance test (OGTT). The course of glycaemic curves in F0 and F1 SHR and SHR-*Zbtb16* adult female rats. F0 SHR control females (dark brown squares, full line), F1 SHR females HSD/HSD programmed (purple empty squares, dashed line), F0 SHR-*Zbtb16* control females (dark red triangles, full line), F1 SHR-*Zbtb16* females HSD/HSD programmed (dark teal empty triangles, dashed line). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and MATERNAL DIET as major factors) Fisher's test of pairwise group comparison within each strain are indicated as follows: * p<0.05, ** p<0.01, *** p<0.001. Strain differences between F0 SHR and SHR-Zbtb16 control females are represented by black asterisks (*), t=60 min *. Effects of programming in SHR females are represented by purple asterisks (*) - significant differences between F0 not programmed and F1 HSD/HSD programmed females, t=0 min ***; in SHR-Zbtb16 females are represented by dark teal asterisks (*) - significant differences between F0 not programmed and F1 HSD/HSD programmed females, t=0 min **.

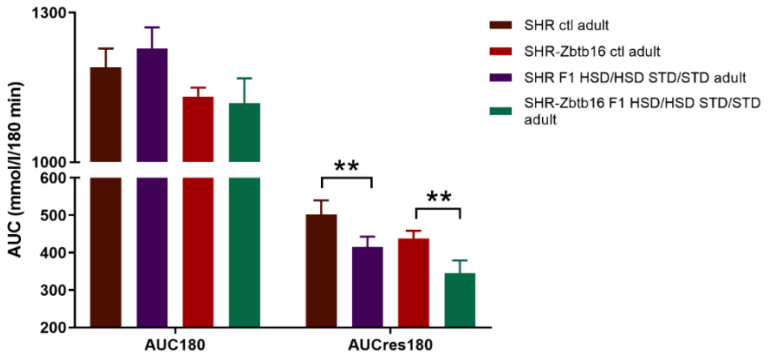


Fig. 52. Area under the curve. Area under the glycaemic curve values calculated from the course of glycaemic curves from 0 to 180 (AUC180) and value of the residual area under the curve (AUCres180). Adult F0 SHR control females (dark brown bars), F1 SHR females HSD/HSD programmed (purple bars), F0 SHR-*Zbtb16* control females (dark red bars) and F1 SHR-*Zbtb16* females HSD/HSD programmed (dark teal bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and MATERNAL DIET as major factors) Fisher's test of pairwise group comparison within each strain are indicated as follows: ** $p<0.01$.

On the 10th day of pregnancy, the OGTT also showed significant differences in blood glucose levels of F1 HSD/HSD programmed females (Fig. 53). In SHR, these changes included lower fasting glycaemia as well as lower blood glucose levels 2 hours after administration of glucose load compared to control. In SHR-Zbtb16, F1 HSD/HSD programmed pregnant females showed lower glucose levels 90 minutes after administration of glucose load compared to control, which reflected in smaller area under the curve as well (Fig. 54).

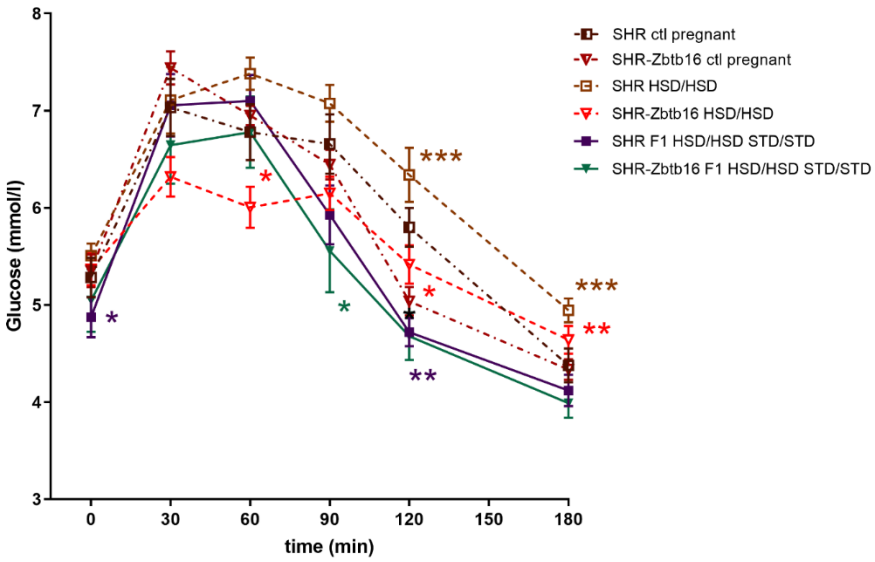


Fig. 53. The oral glucose tolerance test (OGTT). The course of glycaemic curves in F0 and F1 SHR and SHR-*Zbtb16* pregnant female rats, F0 SHR control females (dark brown half empty squares, dashed line), F0 SHR females fed HSD during pregnancy (light brown empty squares, dashed line), F1 SHR females HSD/HSD programmed (purple squares, full line), F0 SHR-*Zbtb16* control females (dark red half empty triangles, dashed line), F0 SHR-*Zbtb16* females fed HSD during pregnancy (red empty triangles, dashed line) and F1 SHR-*Zbtb16* females HSD/HSD programmed (dark teal triangles, full line). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and DIET as major factors) Fisher's test of pairwise group comparison within each strain are indicated as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Effects of programming in SHR females are represented by purple asterisks (*) - significant differences between F0 not programmed and F1 HSD/HSD programmed females, t=0 min *, t=120 min **; in SHR-Zbtb16 females are represented by dark teal asterisks (*) - significant differences between F0 not programmed and F1 HSD/HSD programmed females, t=90 min *. Effects of programming and diet in SHR females are represented by light brown asterisks (*) – significant differences between F0 females fed HSD in pregnancy and F1 HSD/HSD programmed females fed STD in pregnancy, t=120 min ***, t=180 min ***; in SHR-Zbtb16 females are represented by red asterisks (*) – significant differences between F0 females fed HSD in pregnancy and F1 HSD/HSD programmed females fed STD in pregnancy, t=60 min *, t=120 min *, t=180 min **.

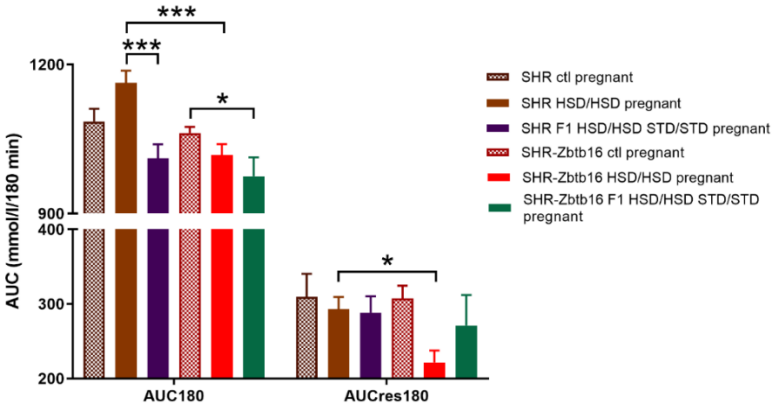


Fig. 54. Area under the curve. Area under the glycaemic curve values calculated from the course of glycaemic curves from 0 to 180 (AUC180) and value of the residual area under the curve (AUCres180). Pregnant F0 SHR control females (dark brown patterned bars), F0 SHR females fed HSD during pregnancy (light brown bars), F1 SHR females HSD/HSD programmed (purple bars), F0 SHR-*Zbtb16* control females (dark red patterned bars), F0 SHR-*Zbtb16* females fed HSD during pregnancy (red bars) and F1 SHR-*Zbtb16* females HSD/HSD programmed (dark teal bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and DIET as major factors) Fisher's test of pairwise group comparison within each strain are indicated as follows: * $p<0.05$, *** $p<0.001$.

5.3.2. F2 offspring

5.3.2.1. Morphometry

Morphometric analysis of F2 HSD/HSD programmed offspring showed significant differences compared to STD/STD not programmed controls. Absolute and relative weights of kidneys in SHR F2 HSD/HSD programmed males were significantly higher than in SHR-Zbtb16 F2 HSD/HSD programmed males (Fig. 55, 56). The relative weights of adrenals have decreased in both strains of F2 programmed males, with lower values in SHR F2 HSD/HSD programmed males (Fig. 57). Both absolute and relative weights of retroperitoneal and epididymal fat pads were higher in SHR F2 HSD/HSD programmed males compared to SHR-Zbtb16 of the same group (Fig. 58, 59). Relative weights of interscapular brown fat were increased in both strains of F2 programmed animals compared to their respective controls (Fig. 59). Muscle mass in m. soleus of the hind leg also increased as a result of F2 generation programming in both strains (Fig. 60).

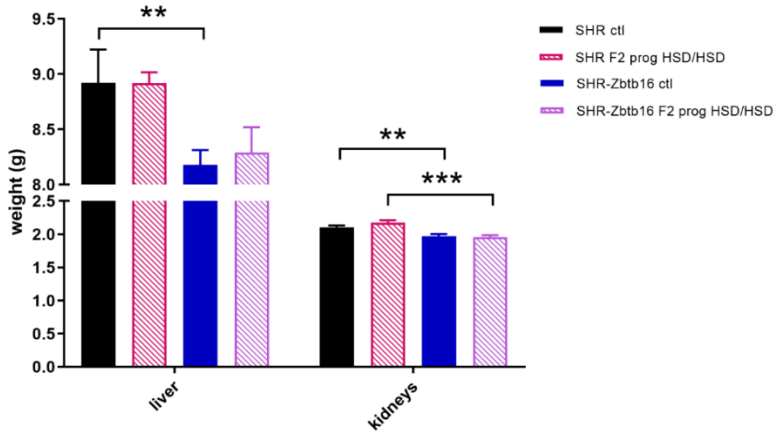


Fig. 55. Liver and kidney weight in adult F1 SHR control males (black bars), F2 SHR males programmed with grandmaternal HSD both in pregnancy and lactation (magenta patterned bars), F1 SHR-Zbtb16 control males (dark blue bars) and F2 SHR-Zbtb16 males programmed with grandmaternal HSD both in pregnancy and lactation (lavender patterned bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and DIET as major factors) Fisher's test of pairwise group comparison (control vs F1 programmed vs F2 programmed males) within each strain are indicated as follows: ** $p<0.01$, *** $p<0.001$.

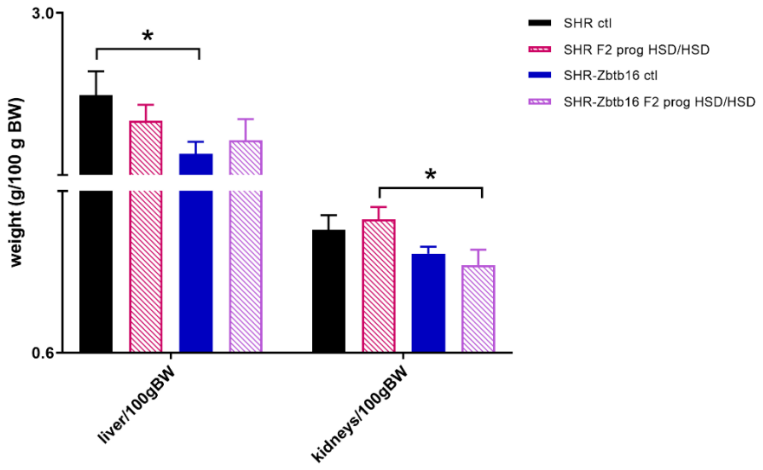


Fig. 56. Liver and kidney weight per 100 g of body weight in adult F1 SHR control males (black bars), F2 SHR males programmed with grandmaternal HSD both in pregnancy and lactation (magenta patterned bars), F1 SHR-Zbtb16 control males (dark blue bars) and F2 SHR-Zbtb16 males programmed with grandmaternal HSD both in pregnancy and lactation (lavender patterned bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and DIET as major factors) Fisher's test of pairwise group comparison (control vs F1 programmed vs F2 programmed males) within each strain are indicated as follows: * $p<0.05$.

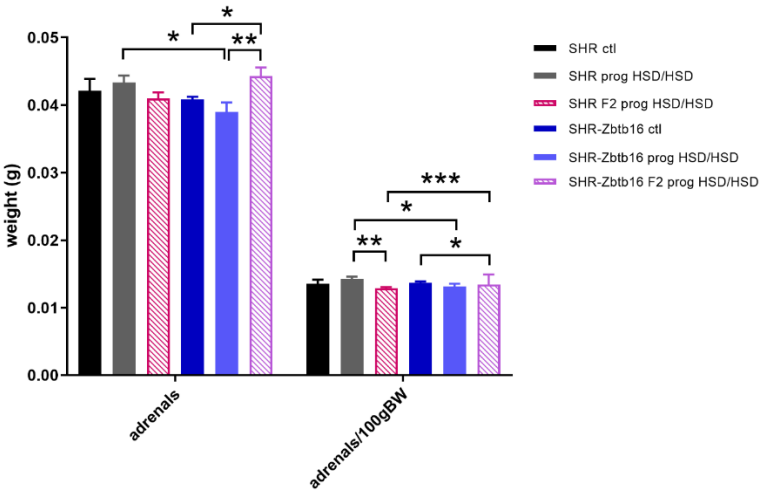


Fig. 57. Adrenal weight per 100 g of body weight in adult F1 SHR control males (black bars), F2 SHR males programmed with grandmaternal HSD both in pregnancy and lactation (magenta patterned bars), F1 SHR-Zbtb16 control males (dark blue bars) and F2 SHR-Zbtb16 males programmed with grandmaternal HSD both in pregnancy and lactation (lavender patterned bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and DIET as major factors) Fisher's test of pairwise group comparison

(control vs F1 programmed vs F2 programmed males) within each strain are indicated as follows: * p<0.05, ** p<0.01, *** p<0.001.

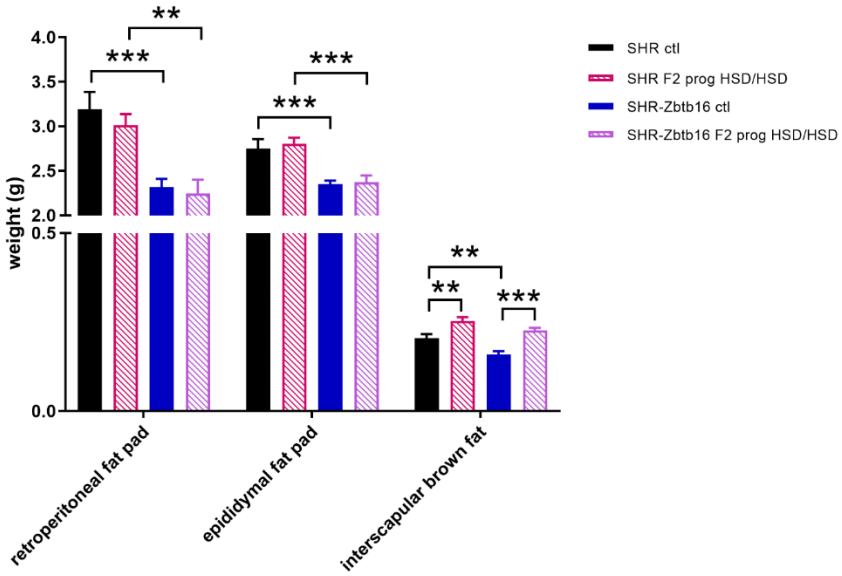


Fig. 58. Retroperitoneal fat pad, epididymal fat pad and interscapular brown fat weight in adult F1 SHR control males (black bars), F2 SHR males programmed with grandmaternal HSD both in pregnancy and lactation (magenta patterned bars), F1 SHR-Zbtb16 control males (dark blue bars) and F2 SHR-Zbtb16 males programmed with grandmaternal HSD both in pregnancy and lactation (lavender patterned bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and DIET as major factors) Fisher's test of pairwise group comparison (control vs F1 programmed vs F2 programmed males) within each strain are indicated as follows: * p<0.05, ** p<0.01, *** p<0.001.

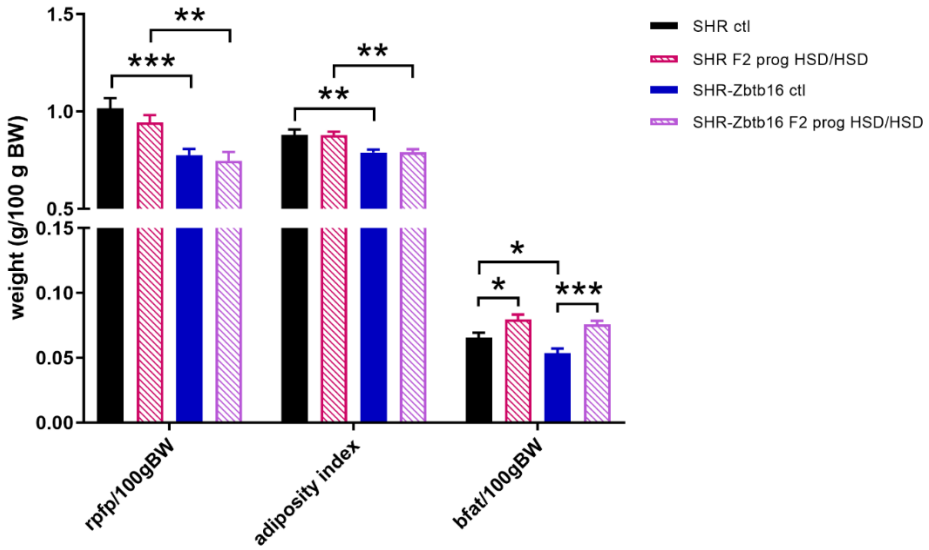


Fig. 59. Retroperitoneal fat pad, epididymal fat pad (adiposity index) and interscapular brown fat weight per 100 g of body weight in adult F1 SHR control males (black bars), F2 SHR males programmed with grandmaternal HSD both in pregnancy and lactation (magenta patterned bars), F1 SHR-Zbtb16 control males (dark blue bars) and F2 SHR-Zbtb16 males programmed with grandmaternal HSD both in pregnancy and lactation (lavender patterned bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and DIET as major factors) Fisher's test of pairwise group comparison (control vs F1 programmed vs F2 programmed males) within each strain are indicated as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

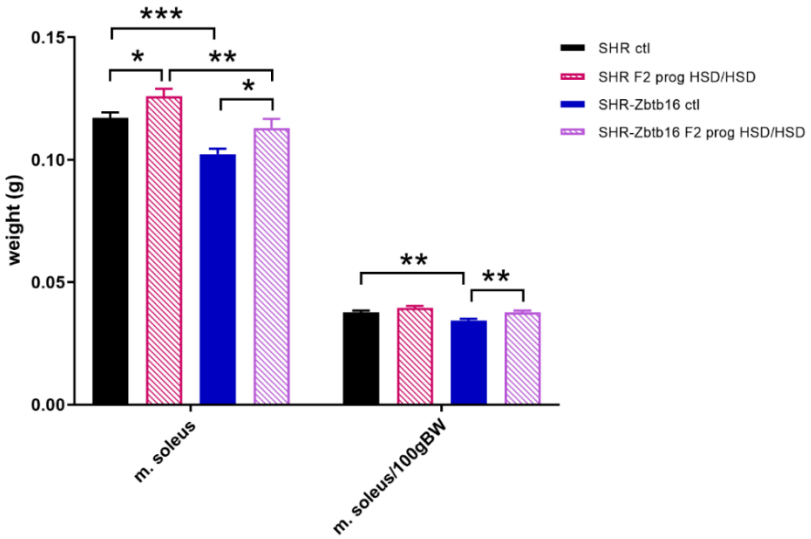


Fig. 60. Hind leg skeletal muscle (musculus soleus) weight in adult F1 SHR control males (black bars), F2 SHR males programmed with grandmaternal HSD both in pregnancy and lactation (magenta patterned bars), F1 SHR-Zbtb16 control males (dark blue bars) and F2 SHR-Zbtb16 males programmed with grandmaternal HSD both in

pregnancy and lactation (lavender patterned bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and DIET as major factors) Fisher's test of pairwise group comparison (control vs F1 programmed vs F2 programmed males) within each strain are indicated as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

5.3.2.2. Glucose tolerance and insulin levels

Fasting glycaemia of SHR-Zbtb16 F2 HSD/HSD programmed males have decreased significantly in adulthood compared to their control (Fig. 61). Blood glucose levels at 90 and 120 minutes after administration of glucose load were significantly decreased compared to the control, which reflected in significantly smaller area under the curve as well (Fig. 62). SHR F2 HSD/HSD programmed males showed no difference in fasting glycaemia, but significantly lower glycaemia 1 hour after the administration of glucose load, compared to their control, but still significantly higher than in SHR-Zbtb16 F2 HSD/HSD programmed males. This trend was apparent 3 hours after administration of glucose load, when SHR F2 HSD/HSD programmed males had higher glycaemia than SHR-Zbtb16 F2 HSD/HSD programmed males, however their glycaemia was significantly higher than glycaemia in controls.

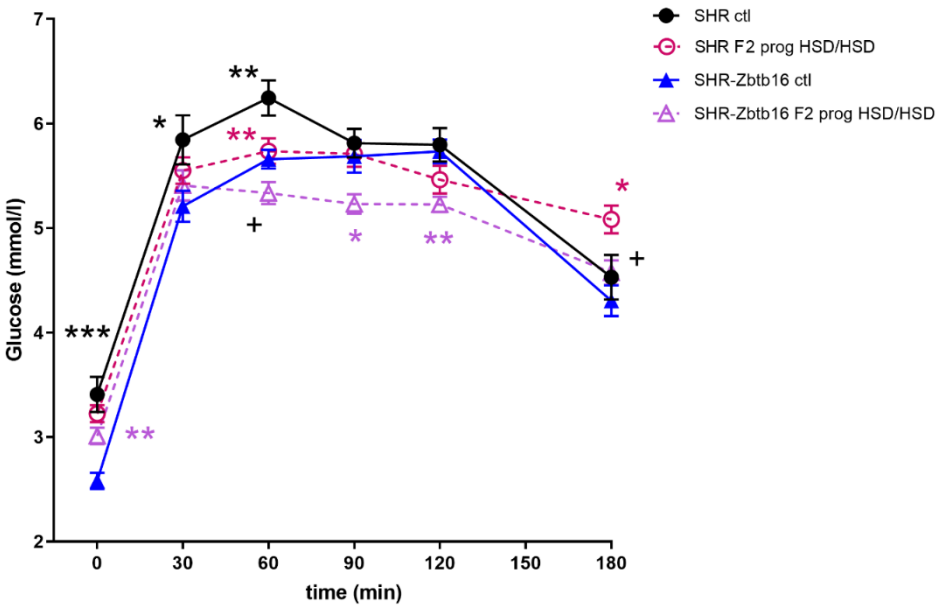


Fig. 61. The oral glucose tolerance test (OGTT). The course of glycaemic curves in adult F1 SHR control males (black circles, full line), F2 SHR males programmed with grandmaternal HSD both in pregnancy and lactation (magenta empty circles, dashed line), F1 SHR-Zbtb16 control males (dark blue triangles, full line) and F2 SHR-Zbtb16 males programmed with grandmaternal HSD both in pregnancy and lactation (lavender empty triangles,

dashed line). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and DIET as major factors) Fisher's test of pairwise group comparison (control vs F1 programmed vs F2 programmed males) within each strain are indicated as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Strain differences are represented as black asterisks (*) – significant differences between F1 SHR control males and F1 SHR-Zbtbt16 control males, t=0 min ***, t=30 min *, t=60 min **; as black plus signs (+) – significant differences between F2 SHR males programmed with grandmaternal HSD and F2 SHR-Zbtb16 males programmed with grandmaternal HSD, t=60 min *, t=180 min *. Effects of programming in SHR males are represented by magenta asterisks (*) – significant differences between F1 control SHR males and F2 SHR males programmed with grandmaternal HSD, t=60 min **, t=180 min *. Effects of programming in SHR-Zbtb16 males are represented by lavender asterisks (*) – significant differences between F1 control SHR-Zbtb16 males and F2 SHR-Zbtb16 males programmed with grandmaternal HSD, t=0 min **, t=90 min *, t=120 min **.

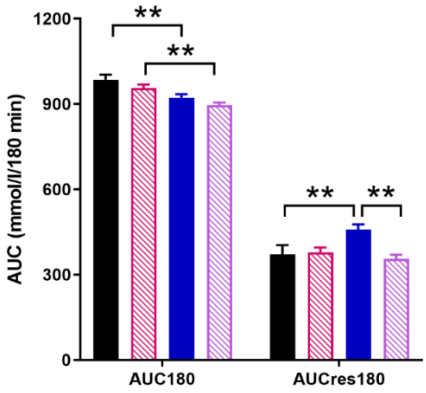


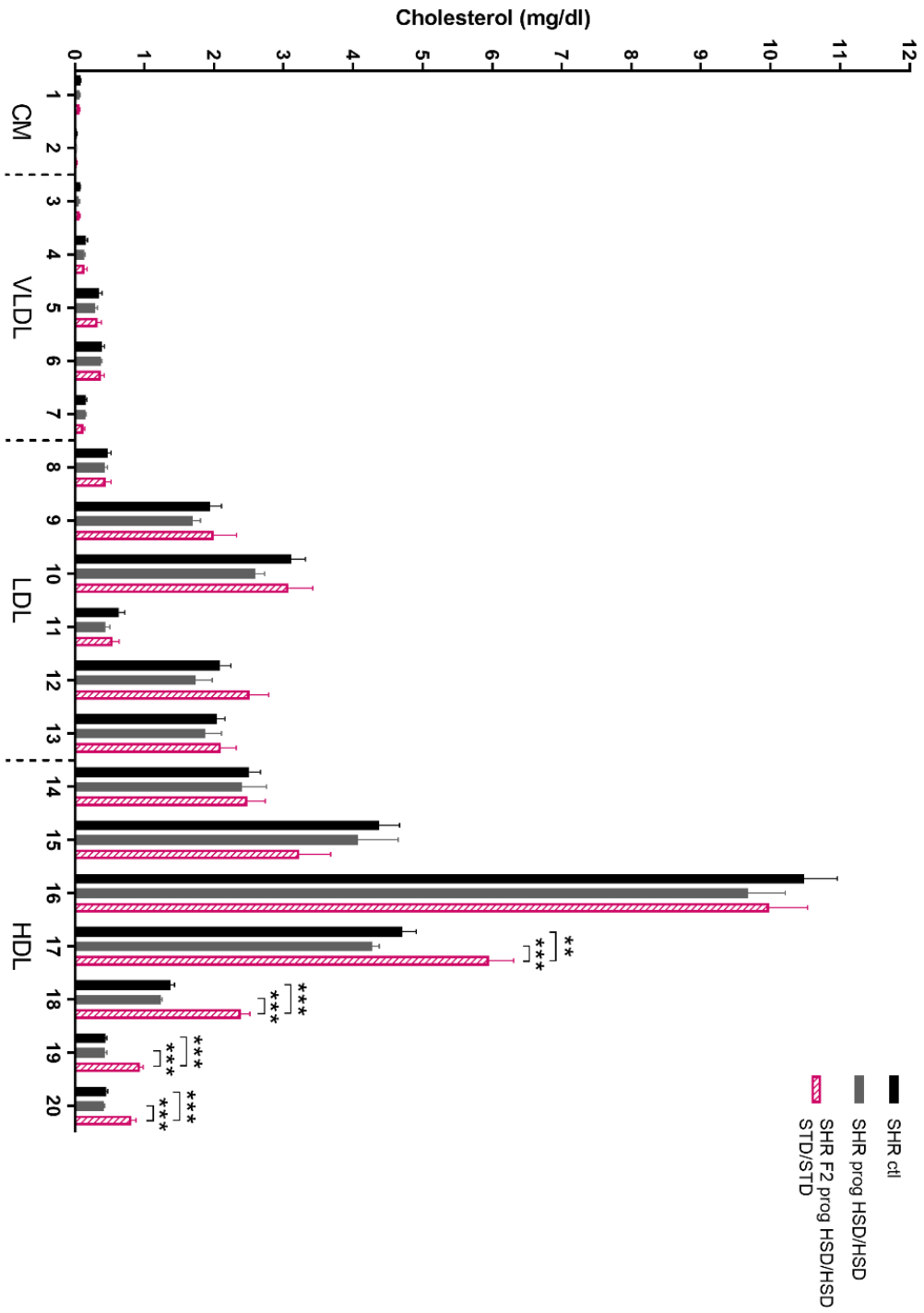
Fig. 62. Area under the curve. Area under the glycaemic curve values calculated from the course of glycaemic curves from 0 to 180 (AUC180) and value of the residual area under the curve (AUCres180). Adult F1 SHR control males (black bars), F2 SHR males programmed with grandmaternal HSD both in pregnancy and lactation (magenta patterned bars), F1 SHR-Zbtb16 control males (dark blue bars) and F2 SHR-Zbtb16 males programmed with grandmaternal HSD both in pregnancy and lactation (lavender patterned bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and DIET as major factors) Fisher's test of pairwise group comparison (control vs F1 programmed vs F2 programmed males) within each strain are indicated as follows: ** $p<0.01$.

5.3.2.3. Lipid profile

The cholesterol profile of F2 HSD/HSD programmed males of both strains showed significant differences compared to the controls (Fig. 63). In both strains, grandmaternal programming with HSD during pregnancy and lactation significantly increased medium, small and very small fractions of HDL cholesterol (SHR control C17 – C20 6.99 mg/dl vs SHR F2 HSD/HSD programmed C17 – C20 10.1 mg/dl, 44.5% increase; SHR-Zbtb16 control C17 –

C20 7.32 mg/dl vs SHR-Zbtb16 HSD/HSD programmed C17 – C20 10.38 mg/dl, 41.9% increase). This significant increase in medium to very small HDL content was accompanied by a significant decrease in particle size of HDL cholesterol in both F2 programmed groups (Fig. 64). In addition, in SHR-Zbtb16 F2 HSD/HSD programmed males we also observed a significant increase in particle size of VLDL.

The triacylglycerol profile of SHR F2 HSD/HSD programmed males showed significant decrease in very small LDL, very large and large HDL subfractions compared to control (Fig. 65). We observed more effects in SHR-Zbtb16 F2 HSD/HSD programmed males, in which the content of very small LDL, very large, large and medium HDL was decreased significantly compared to controls. The particle size of HDL triacylglycerols decreased significantly in both F2 programmed groups again with increase in particle size of VLDL only present in SHR-Zbtb16 F2 HSD/HSD programmed males compared to SHR-Zbtb16 F1 HSD/HSD programmed males (Fig. 66).


Cholesterol (mg/dl)
0
1
2
3
4
5
6
7
8
9
10
11
12
CM
VLDL
LDL
HDL
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
**
***
***
***
***
***
SHR ctl
SHR prog HSD/HSD
SHR F2 prog HSD/HSD
STD/STD

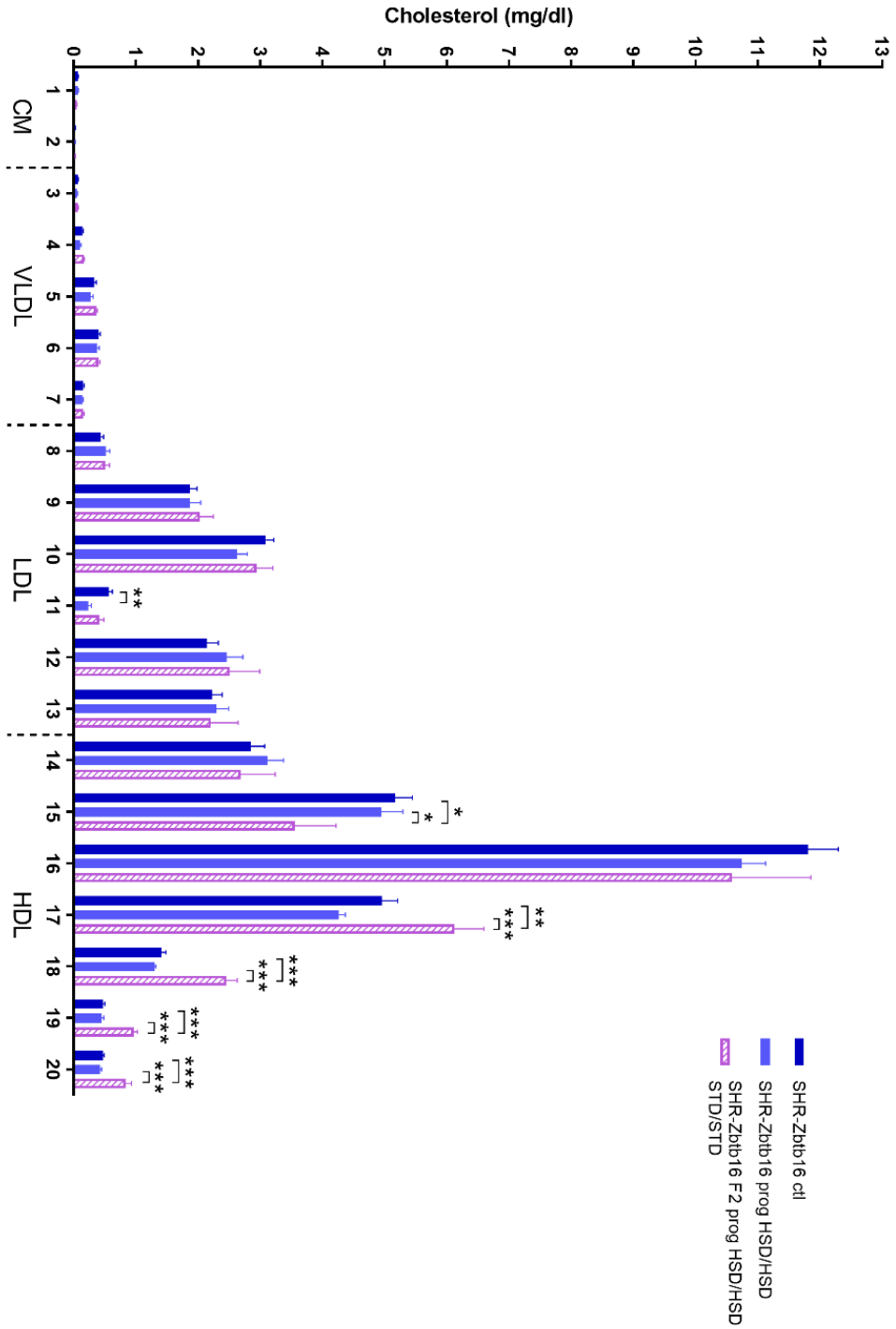


Fig. 63. Cholesterol profile. The cholesterol (C) content in 20 lipoprotein subfractions in adult F1 SHR control males (black bars), F1 SHR males programmed with maternal HSD both in pregnancy and lactation (grey bars), F2 SHR males programmed with grandmaternal HSD both in pregnancy and lactation (magenta patterned bars), F1 SHR-Zbtb16 control males (dark blue bars), F1 SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation (blue bars) and F2 SHR-Zbtb16 males programmed with grandmaternal HSD both in pregnancy and lactation (lavender patterned bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and DIET as major factors) Fisher's test of pairwise group comparison (control vs F1 programmed vs F2 programmed males) within each strain are indicated as follows: *

p<0.05, ** p<0.01, *** p<0.001. The allocation of individual lipoprotein subfractions to major lipoprotein classes is shown in order of particle's decreasing size from left to right. CM-chylomicron, VLDL-very low density lipoprotein, LDL-low density lipoprotein, HDL-high density lipoprotein.

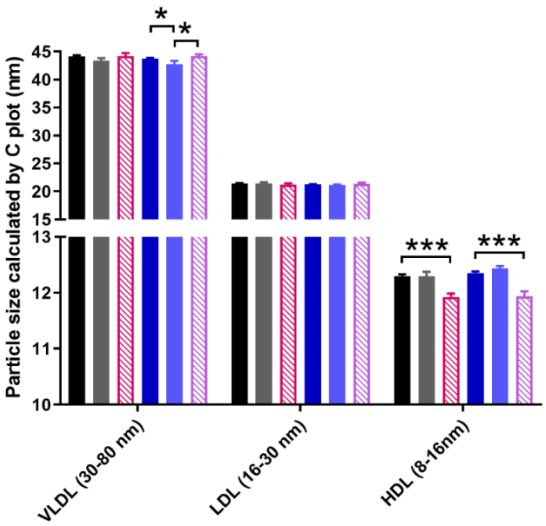
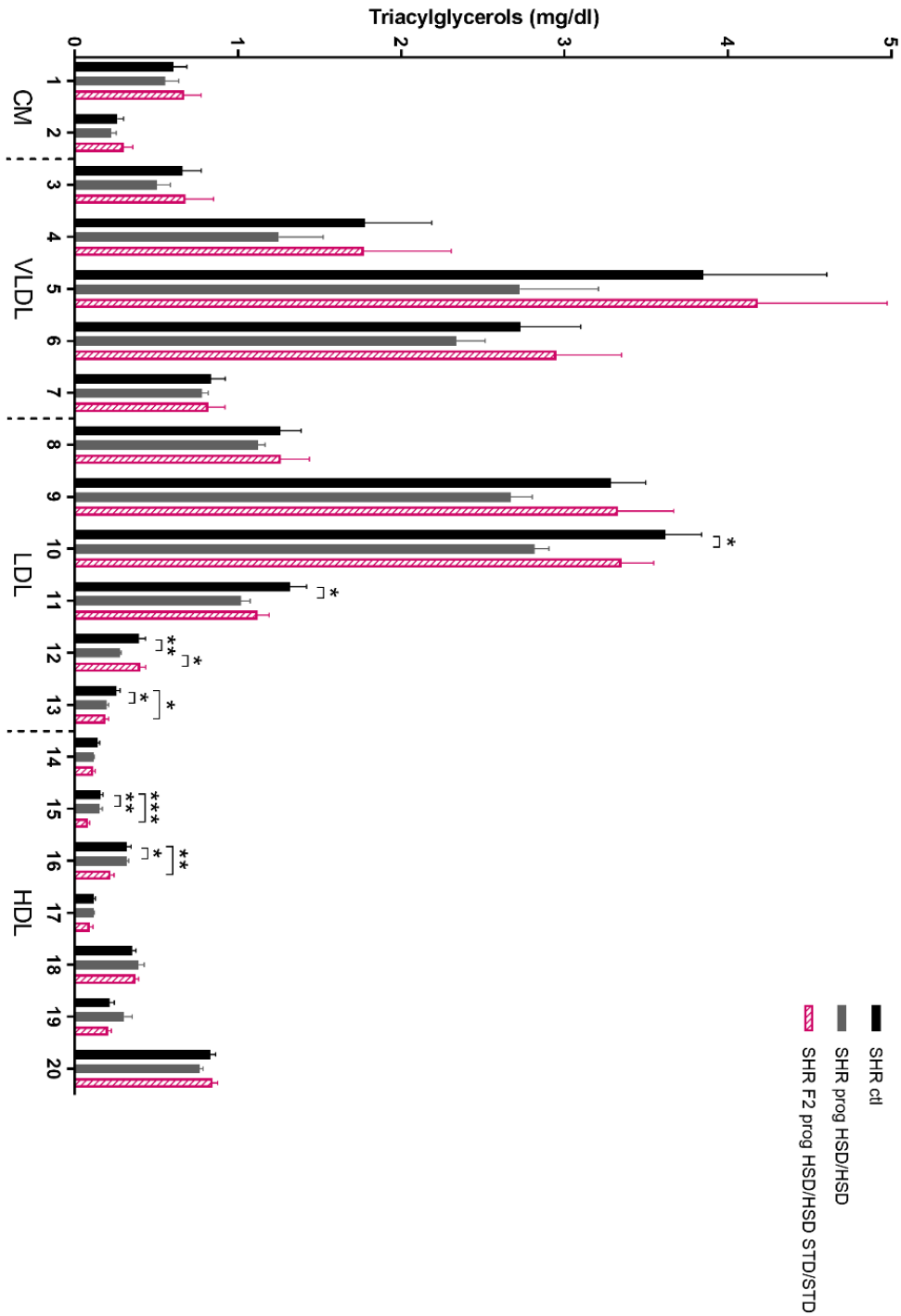


Fig. 64. Major lipoprotein classes particle size calculated by C plot in adult F1 SHR control males (black bars), F1 SHR males programmed with maternal HSD both in pregnancy and lactation (grey bars), F2 SHR males programmed with grandmaternal HSD both in pregnancy and lactation (magenta patterned bars), F1 SHR-Zbtb16 control males (dark blue bars), F1 SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation (blue bars) and F2 SHR-Zbtb16 males programmed with grandmaternal HSD both in pregnancy and lactation (lavender patterned bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and DIET as major factors) Fisher's test of pairwise group comparison (control vs F1 programmed vs F2 programmed males) within each strain are indicated as follows: * p<0.05, *** p<0.001. The allocation of individual lipoprotein subfractions to major lipoprotein classes is shown in order of particle's decreasing size from left to right. VLDL-very low density lipoprotein, LDL-low density lipoprotein, HDL-high density lipoprotein.


Triacylglycerols (mg/dl)
0
1
2
3
4
5
CM
VLDL
LDL
HDL
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
*
*
**
*
*
**
***
*
**
SHR ctl
SHR prog HSD/HSD
SHR F2 prog HSD/HSD STD/STD

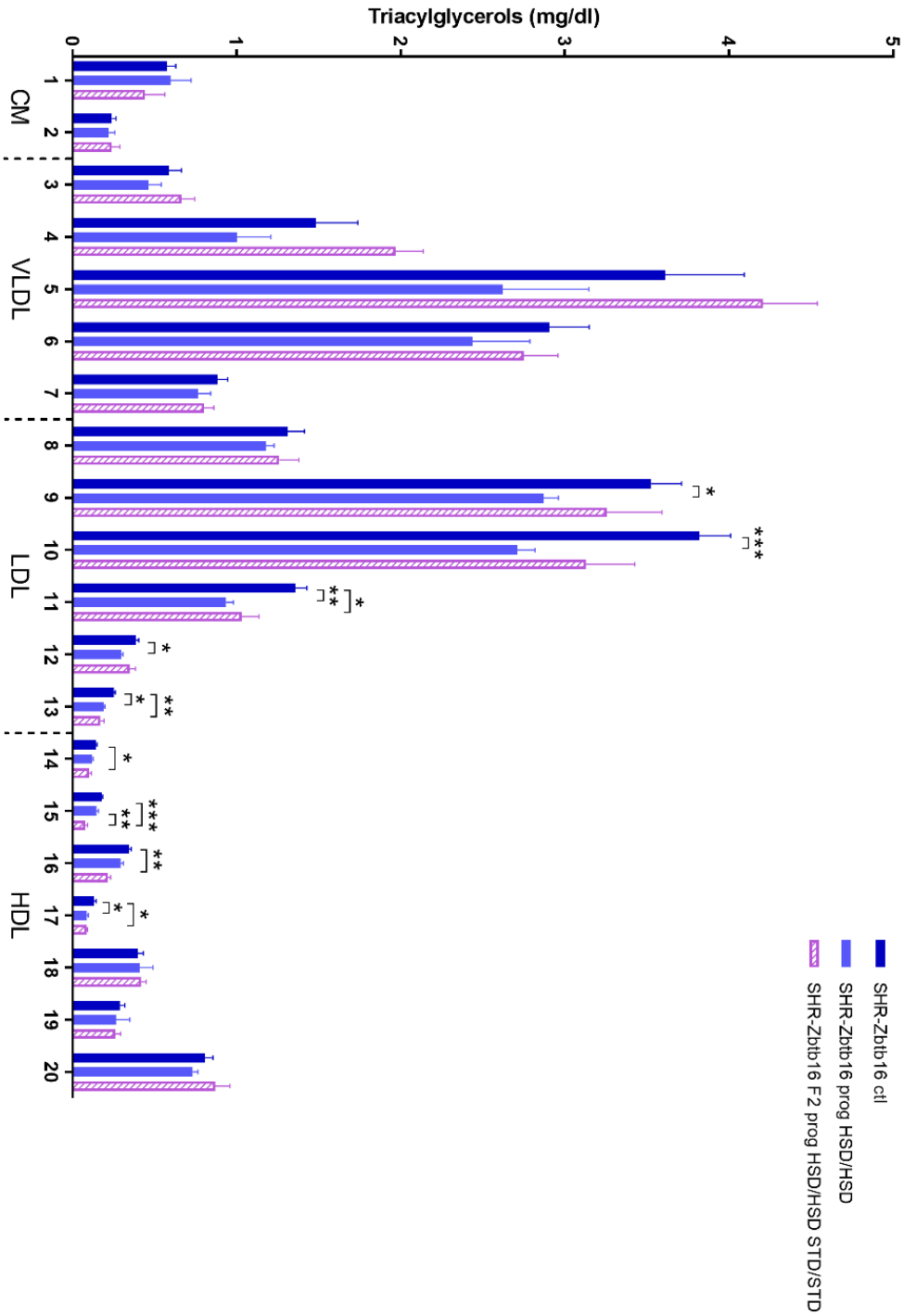


Fig. 65. Triacylglycerol profile. The triacylglycerol (TG) content in 20 lipoprotein subfractions in adult F1 SHR control males (black bars), F1 SHR males programmed with maternal HSD both in pregnancy and lactation (grey bars), F2 SHR males programmed with grandmaternal HSD both in pregnancy and lactation (magenta patterned bars), F1 SHR-Zbtb16 control males (dark blue bars), F1 SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation (blue bars) and F2 SHR-Zbtb16 males programmed with grandmaternal HSD both in pregnancy and lactation (lavender patterned bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and DIET as major factors) Fisher's test of pairwise group comparison (control vs F1 programmed vs F2 programmed males) within each strain are indicated as follows: *

p<0.05, ** p<0.01, *** p<0.001. The allocation of individual lipoprotein subfractions to major lipoprotein classes is shown in order of particle's decreasing size from left to right. CM-chylomicron, VLDL-very low density lipoprotein, LDL-low density lipoprotein, HDL-high density lipoprotein.

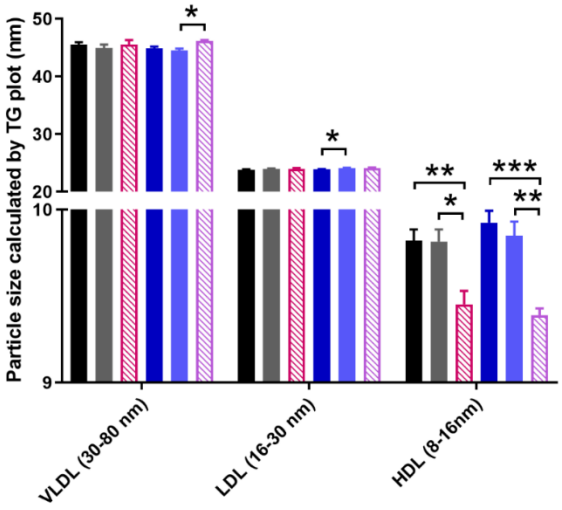


Fig. 66. Major lipoprotein classes particle size calculated by TG plot in adult F1 SHR control males (black bars), F1 SHR males programmed with maternal HSD both in pregnancy and lactation (grey bars), F2 SHR males programmed with grandmaternal HSD both in pregnancy and lactation (magenta patterned bars), F1 SHR-Zbtb16 control males (dark blue bars), F1 SHR-Zbtb16 males programmed with maternal HSD both in pregnancy and lactation (blue bars) and F2 SHR-Zbtb16 males programmed with grandmaternal HSD both in pregnancy and lactation (lavender patterned bars). Data are expressed as mean ± SEM. Within the graph, the significance levels of post-hoc (ANOVA for STRAIN and DIET as major factors) Fisher's test of pairwise group comparison (control vs F1 programmed vs F2 programmed males) within each strain are indicated as follows: * p<0.05, ** p<0.01, *** p<0.001. The allocation of individual lipoprotein subfractions to major lipoprotein classes is shown in order of particle's decreasing size from left to right. VLDL-very low density lipoprotein, LDL-low density lipoprotein, HDL-high density lipoprotein.

5.4. Chapter 4 – unpublished studies

The results of unpublished studies are shown in Příloha 2.

6. Discussion

6.1. Study 1 – F0 mothers and F1 male offspring

This study was conducted in order to test the hypothesis, that HSD feeding during critical windows of early mammalian development influences the offspring postnatal health and its effects could be modulated by genetic factors represented by variant *Zbtb16* gene. The results of the study show, that the administration of HSD to pregnant rat dams significantly affects not only their metabolic profiles but, to an extent, also the metabolic and transcriptomic profiles of their offspring. The effects of modulation of maternal macronutrient consumption have been extensively reviewed recently [295; 296]. Providing pregnant female rats with high-sucrose diet *ad libitum* resulted in higher fasting glycaemia and elevation of serum cholesterol and triacylglycerols in chylomicrons and LDL particles in rat dams of both strains. This is in line with observations of previous studies focused on the consumption of diets high in sugar [297] even after just 10 days, when the metabolic screening was performed. Increase in TAG of HSD-fed groups of rat dams is therefore consistent with the common effects of HSD-feeding. The consumption of HSD was elevated in pregnancy in both strains, most likely due to the high palatability of the diet and sweet taste preference of rats [298]. There was, however, no differential impact on body weight between strains or diet groups during pregnancy until the second week of lactation, when the HSD-fed groups became significantly lighter than STD-fed groups. While most of the so far published studies on effects on maternal programming do not follow maternal weight postpartum, let alone under different dietary conditions, our finding is in agreement with a previously published account on sucrose-fed rat dams [297]. In general, rodent fat deposition is increasing during pregnancy, and with lactation, the storage of lipids in adipose tissue is lowering as lipids are being transferred into milk [299]. The significant reduction of body weight in lactating females fed HSD may be related to altered carbohydrate source in the diet interacting with demands of lactation as a similar drop was observed in rat dams fed a low-protein diet [300]. Indeed, the HSD administration can be envisioned as an enhanced “stress” or metabolic challenge, resulting in worse coping in the early postpartum period [301]. On the other hand, we did not observe any effect on the growth rates between the offspring of HSD and STD-fed SHR and SHR-Zbtb16 dams.

Maternal HSD administration substantially increased brown fat weight in adult male offspring of both strains. It points to an interesting connotation with a study showing that brown

fat is a critical regulator of the effect of maternal nutritional programming [302]. We observed a similar effect in the second generation of both SHR and SHR-Zbtb16 offspring of HSD-fed rat dams (see 5.3.2.1., Fig. 58, 59) [303]. However, the transcriptomic profile in both strains suggests a compromised function of brown adipose tissue. The most enriched canonical pathway in both strains was Mitochondrial dysfunction, and we observed and validated a two-fold increase in expression of hydroxysteroid 11-beta dehydrogenase 1 (*Hsd11b1*), overexpression of which was shown to suppress brown adipocyte function [304]. This is complemented by a significant decrease of Iodothyronine deiodinase 2 (*Dio2*) gene expression, a major activating deiodinase [305]. Most pronounced programming effect across all tissues at the level of individual upstream regulators was inhibition of RICTOR, a regulatory subunit of the mammalian target of rapamycin complex 2. Loss of RICTOR leads to the global dampening of insulin/AKT signaling [306]. Although this observation did not translate to change of global glucose tolerance in the programmed rat offspring, subtler change in insulin resistance of peripheral tissues cannot be excluded, as suggested by the three-fold (two-fold in SHR-Zbtb16) reduction of Glut4 expression in white adipose tissue of SHR male offspring and, in a network perspective, the glucose metabolism disorder activated node. However, it seems that this effect may be secondary to changes in the expression of lipid metabolism-related transcripts, including *Srebf1*, *Srebf2*, *Pcsk9*, *Scd*, *Acat2* and others, observed particularly in livers of both strains. A systematic shift in expression of more than 20 transcripts indicated a substantial downregulation of cholesterol metabolism and synthesis of cholesterol esters. An intriguing result of upregulation of *Lcn2* gene exclusively in the white adipose tissue of SHR-Zbtb16 HSD-programmed males points to a study of Yoo et al., which correlates expression of lipocalin 2 gene in rodent and human visceral adipose tissues with serum levels of lipocalin 2, IL-6 and increased WBC count, which are biomarkers of the inflammatory process [307] linked to metabolic syndrome. Lipocalin 2 is a metabolic syndrome-related adipokine, whose expression was significantly increased in adipose tissue samples of obese animal and human compared to lean controls [307], however, in our study, we observed only one parameter of visceral adiposity (relative weight of retroperitoneal fat pad) to be significantly increased in HSD-programmed SHR-Zbtb16 males. In the absence of obesity, we can speculate, that additional metabolic challenge, such as re-exposure to HSD discussed in 6.2., could potentially shift their metabolic phenotype to metabolic syndrome-like phenotype particularly in HSD-programmed SHR-Zbtb16 animals, as analyzed adipose tissues of SHR HSD-programmed animals didn’t show any significant change in expression of *Lcn2*.

A related result contrasting several previous studies [246; 308; 295] was the slight improvement of the lipid profile of programmed offspring, particularly in the class of LDL-TG, associated with low-grade systemic inflammation and coronary artery disease in humans [309]. Most importantly, we show that the effect of maternal nutritional programming is dependent on the genomic background it acts upon. The variant *Zbtb16* allele present in the SHR-Zbtb16 strain is likely responsible for several subtle distinct effects of maternal HSD on adult male offspring, including less pronounced response of insulin levels and particularly the transcriptome shifts, most apparent in white adipose tissue. *Zbtb16* is a downstream effector for PGC-1-controlled gluconeogenesis, and at the same time, *Zbtb16* negatively regulates the insulin signaling pathway by decreasing the phosphorylation of IRS1, Akt, and FoxO1 in normal mice. Liver-specific knockdown of *Zbtb16* relieved hyperglycemia in db/db mice and led to decreased insulin levels, improved glucose and pyruvate tolerance, and insulin sensitivity [285]. We showed earlier that SHR-*Lx* congenic strain carrying the same 2kb-deletion in an intron of *Zbtb16* as the SHR-Zbtb16 strain displays higher sensitivity to dexamethasone-induced insulin resistance of the skeletal muscle when compared to SHR controls [258] and this effect was persistent in SHR-Zbtb16 itself [310]. The use of only male offspring can be limiting, as sex-specific metabolic syndrome phenotype was previously demonstrated [311] and programming of several related traits is sex-dependent [312]. We focused the study on effects of a minute genetic difference, therefore we opted to use only males of a highly inbred model of metabolic syndrome to maximize the homogeneity of our control and experimental groups and avoid e.g. the potential effects of estrous cycle on gene expression. By assessing the effects of maternal programming in genetically distinct models, it may become possible to elucidate the genetic component of susceptibility to dietary regimens in the early development (Sedova et al., 2007). Also, as this experimental protocol was set up so that HSD was administered throughout the pregnancy and lactation, it is impossible to distinguish which of the potentially critical periods (pre-conception, gestation, lactation) is more influential concerning the observed phenotypic effects. Further studies should address in detail the mechanisms and pathways, through which the *Zbtb16* mediates the distinct programming effect since its expression on the level of mRNA was not changed in our study. Also, without confirmation on a mechanistic level, the transcriptome-derived relationships are only indicative of possible underlying processes that still need to be validated. The presented results show that HSD administration to pregnant rats leads to increase in brown adipose tissue weight and slight reduction of LDL triacylglycerols in their adult male offspring. At the same time, maternal HSD administration triggered substantial, strain-specific shifts in transcriptomes of liver, white and

brown adipose tissues. The variant *Zbtb16* allele led to strain-specific effect of HSD-induced changes in transcriptomic profiles of the offspring with a limited effect on induced metabolic changes.

6.2. Study 2 – F1 male offspring subjected to HSD challenge in adulthood

In study 2 we tested the hypothesis that nutritional challenge with high-sucrose diet in adulthood would exacerbate the different responses in SHR vs. SHR-Zbtb16 animals. The experiment involved adult males of both strains programmed by HSD during early development (F1 programmed HSD/HSD), which were nutritionally challenged in adulthood for 14 days prior to sacrifice. Re-exposure to HSD in adulthood resulted in an increase in visceral and retroperitoneal white adipose tissue mass as well as interscapular brown fat in both strains, although the overall body weight did not differ significantly between strains. With oversupply of glucose, the excess acetyl CoA generated by glycolysis can be converted to triacylglycerols in the cytoplasm of adipocytes and stored as fat [313]. Increase in visceral fat mass is a well-known effect of diets rich in simple carbohydrates. Our findings are consistent with experiments administering isocaloric high-fat diets even throughout a longer period of time, where adult rats fed high-fat diet show increase in adiposity while not being overweight [314; 315]. Relative weights of interscapular brown fat in both groups of re-exposed HSD-programmed males were the highest in comparison to any group of adult males in the studies, with the exception of SHR HSD-programmed males exposed to dexamethasone (see 5.4.1.2.1., Fig. 79). In that matter it seems that HSD was effectively increasing brown fat weight in direct proportion to immediate HSD exposure. We also observed a significant increase in the relative weight of liver in SHR-Zbtb16 re-exposed group only, which indicates possible increase in lipid storage. The reported decrease in kidney weight was however significant only in SHR re-exposed males.

The comparison of glucose tolerance between the strains revealed the effect of HSD, which was particularly prominent in SHR-Zbtb16, showing significant increase in area under the glycaemic curve, despite the rise in fasting insulin. Maternal HSD with re-exposition to the same diet in adulthood led to elevated glycaemia in t = 30, 60 and 90 minutes after glucose bolus administration in SHR-Zbtb16. A significant drop of blood glucose levels at t = 60 and 90 minutes with a rise at t = 120 minutes suggest a biphasic course of OGTT [316] and point to impairment of glucose tolerance of re-exposed programmed SHR males. The reaction pattern

in SHR-Zbtb16 re-exposed males as well as the course of OGTT remained the same compared to their controls, with increase in glycaemia. In this context, we identified STRAIN*DIET interaction at t = 60 ($p = 0.00009$) and 90 ($p = 0.007$) minutes of OGTT: in control groups, SHR showed higher glucose concentration at t = 60 min compared to SHR-Zbtb16, whereas opposite was true for the HSD re-exposed groups. This strain-specific effect could be in part attributable to greater rise in fasting insulin in re-exposed SHR animals (78% compared to 44% increase in SHR-Zbtb16). The particular sensitivity of SHR-Zbtb16 to diabetogenic effect of dexamethasone [310] would suggest otherwise, we have however shown a lack of effect of maternal HSD on STD fed adult SHR-Zbtb16 male offspring [317] on impairment of glucose tolerance. The observed differences in glycaemia course of both groups re-exposed to HSD are most likely attributable to postnatal HSD challenge. While the mechanism connecting the variant *Zbtb16* gene comprising an intronic deletion and one conserved nonsynonymous substitution [318] to the specific nutrigenetic response is not clear, there is prior evidence indicating crucial role of *Zbtb16* in glucose homeostasis, particularly regulating hepatic gluconeogenesis [285]. Dysregulation of peroxisome proliferator-activated γ coactivator 1α (PGC-1α)/glucocorticoid receptor – *Zbtb16* crosstalk may lead to increased or more sustained *Zbtb16* effect triggering greater induction of hepatic gluconeogenesis and more effective impairment of insulin signalling [285]. The activation hypothesis is further supported by observation in heterozygous SHR-Zbtb16 knockout rats showing improved glucose tolerance and increased insulin sensitivity of peripheral tissues [288].

Lipid profile analysis also pointed to more pronounced effect of HSD re-exposure in congenic SHR-Zbtb16 strain. Correspondingly to HSD-programmed males, the re-exposed HSD-programmed males of both strains showed decrease of cholesterol content of LDL particles, however it was more prominent after the HSD challenge. We observed a strain-specific nutrigenetic interaction in a decrease of cholesterol content in very large and large HDL particles, with an increase in most fractions of medium, small and very small HDL particles, which was only significant in SHR-Zbtb16 re-exposed males. The pattern of reaction to HSD exposure was similar between the two strains for chylomicrons and VLDL particles. We observed significant STRAIN*DIET interactions for the smallest LDL particles as their triacylglycerol content decreased in SHR-Zbtb16, but did not change in SHR in response to HSD re-exposure. Rise in triacylglycerol content and a decrease or no change in cholesterol content in response to sucrose-rich diet has been reported [319; 320]. Furthermore, heterozygous SHR-Zbtb16 male rats showed decreased concentrations of total serum and

hepatic cholesterol and triacylglycerol content [288]. *Zbtb16* gene variation was also associated with total and LDL cholesterol levels in a human cohort of Caucasian adults [321]. Given the important role of *Zbtb16* in adipose tissue metabolism [287; 322], it is possible that distinct changes in substrate utilization in the two compared strains resulted in the observed shifts of lipid profiles.

The increase of triacylglycerols in chylomicron particles is consistent with an increase in visceral fat of HSD-programmed re-exposed animals. Although we did not measure the levels of free fatty acids in the serum, we observed significantly higher levels of free glycerol in the serum of HSD-programmed re-exposed groups, which could have originated in hydrolysis of abundant triacylglycerols (lipolysis). Increased fasting serum glycerol is considered one of the most important biomarkers for an increased risk of development of hyperglycaemia and type 2 diabetes [323]. Together with possible glucose metabolism impairment represented by higher levels of blood glucose levels in course of OGTT despite the higher levels of insulin, significantly increased levels of free glycerol in the serum of SHR-Zbtb16 re-exposed males compared to SHR re-exposed males indicate that the congenic strain with variant *Zbtb16* gene was more susceptible to the development of type 2 diabetes. The significant increase in relative liver weight in males of congenic strain also suggests they were possibly inclined to development of non-alcoholic fatty liver disease. Interestingly, the serum glycerol level in HSD/HSD programmed SHR-Zbtb16 animals, which were re-exposed to HSD diet were even higher than the levels in animals exposed to dexamethasone discussed in 6.4.1.

6.3. Study 3 – F1 female offspring and F2 male offspring

Study 3 was performed in order to test the hypothesis that high-sucrose diet feeding effects can possibly be transferred and therefore be apparent in the F2 generation of offspring as well. The STD-fed experimental males in question were grandsons of F0 HSD/HSD fed maternal grandmothers and therefore exhibited the effects of HSD directly influencing the early development of their F1 mothers, which were fed STD postnatally. In other terms, the F2 HSD/HSD programmed males were in contact with the HSD only in the stage of female germline stem cells or oogonial stem cells of their F1 HSD/HSD programmed mother.

Sucrose feeding in pregnancy and lactation of F0 generation rat dams affected metabolic profiles of STD-fed F1 and F2 offspring generations. Particularly in F1 these effects could be

in part, predictive adaptive responses (PARs), which are environmental responses without an immediate benefit to the organism, but rather a distant benefit, e. g., the ability to anticipate the future environment [324] and adapt. PARs presumably evolved to enable organisms to cope with transient changes in the environment and therefore "provide a process by which individuals adapt to their future postnatal environment by restricting their range of possible phenotypes to a narrower spectrum, without changing the genotype [324]". In this study, we observed strain-specific body weight differences in the F1-programmed generation of females prior to pregnancy, during which the distinction disappeared. F1 females of SHR-Zbtb16 strain programmed by HSD in early life were significantly lighter than F1 SHR females with the same programming background. Control and F1-programmed females also differed in their body weight, as the programming by maternal HSD contributed to lower body weight of F1 groups compared to controls. The difference in body weight of controls and F1 groups persisted up to the first week of pregnancy. Interestingly, the body weight of the lactating females did not differ between the groups, as opposed to group of their F0 HSD-fed mothers (see 5.1.1.1., Fig. 5), whose weight dropped significantly in this period [317]. The fasting glycaemia was increased in F1-programmed females in adulthood, which suggests a possible inclination to impaired glucose tolerance. However, the effect of pregnancy on glucose tolerance was comparable between both groups and strains, although SHR F1-programmed pregnant dams showed an additional decrease of blood glucose levels at t = 120 min during OGTT. HSD programming also seemed to alter the appetite of F1-programmed females, as they consumed more of the chow during pregnancy than their control group, which slightly increased their energy intake. This is consistent with previous studies which had shown that sucrose diet induced elevated food intake and appetite in offspring exposed to maternal diet containing fructose, either bound (sucrose) or free fructose in form of high-fructose corn syrup [325; 326].

6-months-old F2-programmed males (F2 HSD/HSD STD/STD) showed differences in fasting glycaemia compared to their controls. SHR-Zbtb16 F2-programmed males had significantly higher levels of fasting blood glucose compared to their controls. The effect of programming of their mothers acted differently upon genomic background of each strain, as SHR-Zbtb16 F2-programmed males had generally lower blood glucose levels than SHR F2-programmed males, which showed significantly higher glycaemia 3 hours after glucose bolus administration compared to their controls and to F2 SHR-Zbtb16 males. Selected F2-programmed SHR-Zbtb16 males also showed a significant drop of blood glucose levels at t = 60 min and therefore inclined to biphasic course of OGTT [316], which was not significant in

analysis of a larger subset of animals. Decrease of small to very small LDL triacylglycerols in F2-programmed groups, with more prominent effect in SHR-Zbtb16 was similar as it was in F1-programmed males (see 5.1.2.3., Fig. 27), who directly interacted with HSD through the uteroplacental system and breast milk of F0 mothers [317]. The persistence of this specific decrease seems to be determined by the HSD effect even in second generation of offspring. A similar pattern was observed in decrease of medium HDL triacylglycerols which was more prominent in SHR-Zbtb16 F1-programmed males. In addition, we observed a decrease of very large HDL triacylglycerols in F2-programmed males. Interestingly, levels of medium to very small HDL cholesterol particles have been increased significantly (by 44.5% in SHR and by 41.9% in SHR-Zbtb16) only in F2-programmed males, with simultaneous decrease in size of these particles.

The maternal HSD-specific milieu possibly programmed the offspring for the environment providing high amounts of sucrose, however in adult life their main source of carbohydrates from STD was starch. Predictive adaptive hypothesis postulates, that environmental mismatch of early life versus adulthood can increase the risk of disease [327; 328]. However, historically, it was poor maternal nutrition in early development versus overnutrition in adulthood due to westernized diet popularity that was the most studied model of disparity. Our study established the opposite conditions, whereby maternal diet of F0 generation abundant in sucrose, although the same in calorie content as STD, is followed by STD consumption after weaning of their F1 offspring and whole pre- and postnatal life of their grandsons. If the strategies for maximizing the postnatal survival success are based on the anticipation of a particular adult environment, it is possible that the metabolic systems of F1 and F2 offspring were prepared to manage increased levels of sucrose and thus overproduction of triglycerides and responded with a higher baseline for HDL production in order to alleviate these effects. Triglycerides are being transferred from VLDL to HDL by the action of cholesterol ester transfer protein [329]. After hydrolysis by hepatic lipase they are cleared from plasma, which serves as a basis for protective effect of HDL against dyslipidaemia and coronary heart disease. Another possible explanation for such effects can be derived from capacity-load model proposed by Wells [239; 238], described in more detail in Literature review (see 3.3.2.3.4., Fig. 1). When F0 gestating female is exposed, the metabolic capacity of F2-programmed offspring can be affected by HSD *in utero*, as F2 germline is already present in directly exposed F1 embryo [57]. Furthermore, the specific HSD-programmed metabolic setting of F1 mothers alone has the ability to influence metabolic capacity or capacity for

homeostasis in their offspring, which we showed in Study 1. We can hypothesize, that metabolic capacity of F2-programmed offspring was increased, perhaps intertwined with prediction of HSD-rich adult environment. The low metabolic load provided in adult life of these F2-programmed offspring was a consequence of low environmental exposure to sucrose and therefore mismatched the predicted sugar-abundant situation. As Wells' model proposes, the associations with disease risk in later life are inversely dose responsive. With high metabolic capacity and low metabolic load, their risk for disease remained fairly low, which can be represented by lower levels of LDL triacylglycerols as well as higher levels of HDL. The capacity of F2-programmed offspring to maintain homeostasis was possibly much higher than given postnatal load.

Another effect of HSD-programming in early life that persisted two generations of offspring was a significant increase of interscapular brown fat weight. We already noticed this effect in F1-programmed adult males of both strains (see 5.1.2.1., Fig. 20, 21), although direct programming with HSD resulted in higher increase in F1-programmed males [317] than the increase in second generation [303]. As shown lately, brown fat represents an essential regulator of the effects of maternal nutritional programming [302]. Body fat distribution in terms of retroperitoneal fat and epididymal fat, partly correlating with human visceral fat [330], wasn't affected by the second-generation programming. Relative weights of retroperitoneal fat differed among the strains in F2 generation of programmed males, with SHR having higher relative weights. Strains used in this study differed in a single gene mutation – 254 kb deletion in intronic region of *Zbtb16* gene [274] in order to show that SHR genomic background can exacerbate the effects of *Zbtb16* gene involved in pathogenesis of metabolic syndrome [269]. Conversely, it was shown to improve the related parameters in previous studies [288], which was to an extent apparent in the present one as well. The effects of HSD on metabolic programming can therefore be similar, impaired glucose tolerance or increase of metabolically active brown fat, but they could potentially present themselves differently in each generation, such as increasing the levels of protective HDL cholesterol. In conclusion, the observed effects in F2-programmed generation, labelled as intergenerational when being transferred through the maternal line [57; 331], were attributable to HSD feeding of F0 gestating and lactating females.

6.4. Unpublished studies

6.4.1. Pharmacological challenge – exposure to dexamethasone

Glucocorticoids have a wide range of therapeutic uses and are one of the most widely prescribed drugs. Autoimmune, allergic and inflammatory diseases are often treated with dexamethasone and prednisolone. Unfortunately, their use is limited by wide range of side effects, as they impair insulin sensitivity, cause metabolic abnormalities, affect fat deposition and hypertension [332]. The SHR-Zbtb16 male rats without any metabolic programming background (STD/STD) exposed to dexamethasone in adulthood have shown effects matching this knowledge. The relative weights of heart, liver, kidneys and brown fat were increased in comparison to their untreated controls. Relative weights of adrenals were, however, decreased, which is consistent with known effects of glucocorticoid treatment. Exogenous corticosteroids suppress adrenal function, as they suppress the HPA axis by decreasing corticotropin-releasing hormone synthesis and in connection, the release of adrenocorticotropic hormone. In the absence of adrenocorticotropic hormone, the adrenal cortex loses the ability to produce endogenous cortisol [333]. The increase in fasting insulin levels and fasting glycaemia, accompanied by significantly higher levels of blood glucose 30 and 60 minutes after administration of glucose load are also consistent with previous findings [310]. Interestingly, we observed a significant drop in blood glucose levels of dexamethasone-exposed male rats 2 hours after the administration of glucose load, further pointing to a derangement in glucose tolerance. Exposure to dexamethasone also significantly increased cholesterol and triacylglycerol content in all classes except for LDL particles, which is similar to the effects reported previously [310]. As mentioned in 6.2., the levels of serum glycerol were higher in dexamethasone-treated males compared to untreated controls, yet they were surprisingly lower than in F1 HSD/HSD programmed animals re-exposed to HSD, although not significant.

Treatment with dexamethasone elicited similar responses in F1 HSD/HSD programmed SHR males in the sense of morphometric changes and lipid profile shifts apparent in almost all measured fractions. The course of OGTT resembled the one analysed in STD/STD SHR-Zbtb16 males, even though the drop in blood glucose levels at t = 120 min was not significant. The levels of blood glucose were much lower in programmed SHR males than unprogrammed SHR-Zbtb16, with a striking difference in their fasting insulin levels. The insulin levels of both dexamethasone-treated groups increased compared to their untreated controls, however, HSD/HSD programmed SHR males had twice as higher levels than STD/STD SHR-Zbtb16 males, which similar to the effect we reported in F1 HSD/HSD programmed SHR males re-exposed to HSD described in 6.2. A distinction of this magnitude opens up a question somehow

of what represents worse glucose tolerance – higher glycaemia with lower insulin levels, or comparably lower glycaemia with increased insulin levels moving towards insulin resistance? The glucose tolerance of both groups was undoubtedly impaired as a result of dexamethasone treatment and in the absence of corresponding groups we can only speculate that either presence or absence of maternal HSD programming influenced the response of the two strains.

6.4.2. SHR-Zbtb16 mothers fed STD/HSD and its effect on offspring

The study of programming effect of HSD restricted to postnatal period in SHR-Zbtb16 animals was performed in order to bring more insight into which time in mammalian development is more critical in terms of its sensitivity to maternal diet. Rat dams fed STD during pregnancy and HSD during breastfeeding displayed a trend of lower body weights than STD-fed control in this period, although the difference was not statistically significant. As discussed earlier, the body weights of dams fed HSD in pregnancy as well decreased significantly since second week of lactation compared to STD-fed controls [317] and STD/HSD fed dams in third week of lactation.

The male offspring (F1 STD/HSD) were compared to relevant groups of SHR-Zbtb16 in order to evaluate the effects of HSD restricted to lactation period. The comparison to control and F1 HSD/HSD programmed SHR-Zbtb16 males revealed that developmentally programmed increase in relative weights of interscapular brown fat was in fact comparable to SHR-Zbtb16 offspring, who were influenced by HSD also in prenatal period [317]. The observed shifts in lipid profiles were also comparable to those in F1 HSD/HSD programmed males discussed in 6.1. F1 STD/HSD males had significantly higher glycaemia 3 hours after administration of glucose load compared to controls and HSD/HSD programmed males [317].

Nutritional challenge in adulthood revealed more insight to the role of HSD-programmed background it acted upon. In comparison to HSD/HSD programmed males re-exposed to HSD (see 6.2.), the STD/HSD programmed males re-exposed to HSD did not show significant differences in blood glucose levels during the course of OGTT. As reported recently [334], HSD/HSD programming profoundly impacted the capacity of male rats to tolerate glucose after a nutritional challenge with HSD, increasing the glycaemia despite higher levels of insulin. This points to the notion, that prenatal period is presumably more important of the two periods of early life, in the in respect of developmental metabolic programming with diet.

The observed gradual increase in CM fraction of TAG were in direct proportion to the level of exposure to HSD in early life, i.e. the levels of CM in F1 HSD/HSD programmed animals re-exposed to HSD were the highest, as were the levels of serum glycerol indicating these animals were more prone to development of metabolic syndrome-like phenotype.

In choosing to observe the effects of different maternal programming in context of HSD challenge, we discovered even more compelling results. The baseline for this comparison involved not programmed, STD/STD SHR-Zbtb16 males, which were exposed to HSD only in adult life. We observed the relative weights of kidneys and adiposity to be in indirect proportion to the increasing maternal exposure to HSD, following HSD challenge. The male offspring of mothers fed STD/STD exposed to HSD in adulthood had the highest relative weights of kidneys, followed by the STD/HSD programmed males with the lowest in HSD/HSD programmed males. The reduction in kidney weight is an unexpected result of HSD programming followed by a HSD challenge in adulthood, as increased fructose intake is generally associated with renal glomerular hypertrophy [248-250]. Recent study evaluating the effects of fructose in maternal diet on health of neonate pups till the age of 2 weeks has, however, reported similar results [335] as our own, even though this effect was observed in much older animals in our study. Dietary fructose is metabolized in the liver and increases the production of uric acid, but also reduces uric acid excretion through the kidneys [336]. Reduction in excretion of uric acid is associated with insulin resistance and subsequently diabetic phenotype [336]. It is possible that the observed decrease in renal weights in proportion to the level of metabolic programming with HSD following HSD challenge is connected to kidney impairment to a certain extent.

The female offspring (F1 STD/HSD) programmed with HSD only postnatally showed lower body weights in adulthood and in course of pregnancy/lactation than STD/STD controls, which is similar to our finding of lower body weights in F1 HSD/HSD females (see 5.3.1.1., Fig. 48) [303]. F1 STD/HSD programmed females were nonetheless lighter at the same age than F1 HSD/HSD programmed females too and also showed lower levels of blood glucose in the middle of OGTT. The appetite of F1 STD/HSD programmed rat dams was increased correspondingly to F1 HSD/HSD programmed rat dams, which implies, that the satiety signal control can be influenced by maternal HSD in postnatal period as well [337]. The increase in chow intake was not influenced by sweet taste preference as in HSD-fed dams [317], as F1 STD/HSD programmed dams were fed STD exclusively throughout their adult life and

pregnancy. The F2 offspring (F2 STD/HSD STD/STD), which were programmed only by grandmaternal HSD after delivery, had the same inclination in retaining the elevated blood glucose levels 3 hours after the glucose load administration, as F2 HSD/HSD STD/STD animals [303], despite their mothers and themselves only being fed STD throughout life.

6.4.3. SHR programmed with maternal and grandmaternal HSD

In comparison to all of the other pregnant female groups, the group of F1-programmed HSD/HSD HSD/HSD rat dams consumed the highest amount of chow. The increased appetite baseline of F1-programmed HSD/HSD rat dams was increased even more with the introduction of HSD diet during their own pregnancy. Given the known link between fructose intake and depression of satiety signals [337], it is possible to associate the increased appetite in rat dams to the bound form of fructose in the HSD, which they have been the most exposed to.

The F2-programmed HSD/HSD HSD/HSD males - the group of animals that were programmed with the highest amount of HSD (maternal grandmother HSD/HSD in pregnancy and lactation, mother HSD/HSD in pregnancy and lactation) had significantly increased relative heart mass and decreased relative weight of kidneys compared to only "grandmaternally" programmed F2 HSD/HSD STD/STD males. Studies have shown, that high-fat and high-sucrose diets lead to metabolic heart disease with left ventricular hypertrophy and diastolic dysfunction [338; 339]. Recent study in mice also reported that mice fed high-fructose diet developed cardiac hypertrophy mediated by mitochondrial oxidative stress [340]. The SHR's known predisposition to hypertension can perhaps exacerbate the effects of HSD programming in this extent, manifesting as increased heart mass, even though we did not employ the measurement of oxidative stress. The decrease of renal weight, despite the known effects of fructose on renal hypertrophy [335], was discussed earlier (see 6.4.2.). Relative weights of interscapular brown fat in F2 HSD/HSD HSD/HSD males were comparable to F1 HSD/HSD programmed males. As noted in 6.2, it seems that direct exposure to HSD is a crucial factor in increased brown fat mass, as the F1 HSD/HSD programmed males had the highest weights of interscapular brown fat compared to their controls after the HSD re-exposure, as well as compared to F2 HSD/HSD HSD/HSD programmed males, which were programmed with the highest amount of HSD, but only indirectly.

7. Conclusions and impact of the dissertation thesis

Metabolic syndrome is a common impairment of metabolism with many possible contributing factors. One of them, the effect of environment experiences during early development, acts through parental systems and is of utmost importance in recent research. In this dissertation thesis, I've used extensive methods of analysis to determine the effects of HSD acting during prenatal and early postnatal period in adulthood of the rat offspring, or even subsequent generations.

Alternative carbohydrate source in maternal diet during early development resulted in modification of various metabolic parameters of adult male rats. Sucrose, as the main carbohydrate source in maternal diet, elicited programmed responses in liver and adipose transcriptome as well, which points to the importance of source, not just the amount of macronutrients in maternal diets. Main findings in HSD-programmed adult offspring include increase in proportion of brown fat and inclination towards reduced insulin response based on shifts in gene expression profiles of metabolically active tissues. These findings were extended in studies of F1 offspring postnatally challenged with HSD, which showed that the response to HSD in adulthood is strain-specific, even though model strains SHR and SHR-Zbtb16 differed only in small intronic deletion in *Lx* allele. The metabolic programming combined with nutritional challenge in adulthood exacerbated the decreased capacity of SHR-Zbtb16 animals to cope and as a result they displayed metabolic syndrome-like phenotype represented by dyslipidaemia and propensity to development of type 2 diabetes. Leading insights gained from the study of rats in subsequent generation revealed, that the effects of HSD during sensitive periods of development are successfully passed through the maternal line and can even contrast the effects present in F1 generation. Elevation of fasting glycaemia and HDLs in F2 generation were novel effects, which suggest that sucrose present grandmaternal diet has the capacity to trigger different responses in different generations.

The results of this dissertation project extend the current knowledge regarding the alterations in maternal macronutrient sources and their capability to cause long-life health complications in offspring. We showed, that these changes can be in fact transferred to their progeny, which can cause a multi-generation cascade of impaired metabolic function, presenting itself differently in each generation. Furthermore, the dietary composition and the

source of carbohydrates in maternal diet can influence the development of metabolic disorders in respect to the genetic background. We present new findings about novel congenic strain SHR-Zbtb16, deepening the knowledge regarding possible modulation of the developmental programming effects by variant *Zbtb16* gene. Concurrently with contemporary scientific research, implications of these findings can be a contribution to outlining of prenatal care and nutrition recommendations, improving the quality of life and preventing health complications.

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9. Publication list

Publications, which serve as grounds for this thesis:

Školníková, E., Šedová, L., & Šeda, O. (2020). Grandmother's Diet Matters: Early Life Programming with Sucrose Influences Metabolic and Lipid Parameters in Second Generation of Rats. Nutrients, 12(3), 846. https://doi.org/10.3390/nu12030846. **IF = 4.546**

Školníková, E., Šedová, L., Liška, F., & Šeda O. (2020). SHR-Zbtb16 minimal congenic strain reveals nutrigenetic interaction between Zbtb16 and high-sucrose diet. Physiological Research. 16;69(3):521-527. https://doi.org/10.33549/physiolres.934423. **IF = 1.655**

Školníková, E., Šedová, L., Chylíková, B., Kábelová, A., Liška, F., & Šeda, O. (2020). Maternal High-Sucrose Diet Affects Phenotype Outcome in Adult Male Offspring: Role of Zbtb16. Frontiers in Genetics, 11, 529421. https://doi.org/10.3389/fgene.2020.529421. **IF = 3.258**